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Antioxidant properties of dipyridamole as assessed by chemiluminescence

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The ability of dipyridamole (DIP) to scavenge oxygen metabolites generated by either activated human neutrophils (PMNs) or cell-free systems using luminol(s)- and lucigenin-enhanced chemiluminescence was investigated. In the presence of DIP (15–50 μM) a dose-dependent inhibition period was seen in phorbol myristate acetate (PMA)-stimulated PMNs as assayed by isoluminol-enhanced chemiluminescence (ILCL) with horseradish peroxidase (HRP). Although such a lag period was not observed in the absence of HRP, 50 μM DIP inhibited extracellular ILCL by more than 50%. Intracellular luminol-enhanced chemiluminescence (LCL) as assayed in either PMA- or in ionomycin-activated PMNs was not affected by dipyridamole (15–50 μM). In cell-free systems, DIP produced concentration-dependent inhibition in H_2O_2-(45% at 50 μM), OH- (40%, at 0.1 μM) and HOCl-(20% at 10 μM). Both absorbance and fluorescence scans revealed that DIP is able to react with equimolar quantities of either H_2O_2 or HOCl. These results suggest that DIP scavenges reactive oxygen species (ROS) presumably secreted by activated human PMNs in the following decreasing order: $^{\bullet}OH > HOCl > H_2O_2 \gg O_2^{\bullet-}$.

1. Introduction

The antioxidant properties of dipyridamole (DIP) are generally known [1, 2]. Our approach to the further study of such properties combines physicochemical and photobiological *in vitro* testing on neutrophils. The selection of this type of cell for the study of the antioxidant properties of this vasodilator [3] is explained as follows.

When polymorphonuclear neutrophils (PMNs) are activated *in vitro* by a number of different soluble and particulate stimuli including chemoattractants (e.g., formyl-methionyl-leucyl-phenylalanine, fMLP), phorbol esters (e.g., phorbol myristate acetate, PMA), calcium ionophores (e.g., ionomycin) and serum opsonized zymosan, reactive oxygen species (ROS) are released as a part of a metabolic process called respiratory burst [4]. The biochemical basis of the respiratory burst is the activation of a membrane-bound enzyme system, which catalyzes the one-electron reduction of oxygen to superoxide radical anion ($O_2^{\bullet-}$) at the expense of NADPH. Although $O_2^{\bullet-}$ can dismutate to give hydrogen peroxide (H_2O_2), some studies have indicated that H_2O_2 can be formed *via* a direct two-electron reduction, depending on the pH and NADPH concentration [5]. In addition to the activation of the NADPH oxidase system, PMNs liberate other enzymes such as myeloperoxidase (MPO) which kill microorganisms by oxidizing Cl^- to hypochlorous acid (HOCl). Furthermore, the physiological production of singlet oxygen ($^1O_2$) has been demonstrated in the MPO–H_2O_2–Cl^- system at neutral pH [6]. Some of the above mentioned ROS can excite various chemiluminescent species such as luminol (5-amino-2,3-dihydrophtalazine-1,4-dione) and lucigenin (bis-*N*-methylacridinium nitrate), which thereafter release energy in the form of light (chemiluminescence). Indeed, there is a close link between the luminol-enhanced chemiluminescence (LCL) response and ROS generated during the respiratory burst [6]. However, qualitative and quantitative aspects of ROS generation by PMNs differ significantly from one stimulus to another. For example, it has been proposed that fMLP-stimulated LCL of PMNs predominantly reflects the generation of hydroxyl radical ($^{\bullet}OH$) while PMA-stimulated LCL mostly reflects the formation of hypochlorite (OCl^-) and/or singlet oxygen ($^1O_2$) [7]. On the other hand, lucigenin-enhanced chemiluminescence is widely used as a unique assay for superoxide ($O_2^{\bullet-}$) formation in activated PMNs [8].

Dipyridamole (DIP) is a coronary vasodilator otherwise called 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido [5,4*d*]-pyrimidine. This drug has been used in coronary heart attacks [3] and also exhibits a potent biological antioxidant activity' behaving as an inhibitor of lipid peroxidation [1] as well as a scavenger of superoxide, hydroxyl and peroxyl radicals [2]. Furthermore, because of its lipophilic cationic character, DIP is one of the substances that can inhibit P-glycoprotein–mediated drug efflux and thus reverse multidrug-resistance (MDR) to chemotherapeutic agents [9]. On the other hand, we have shown in a recent report [10] that DIP is phototoxic when assayed by the photohemolysis method on human erythrocytes and on linoleic acid in a lipid peroxidation model at concentrations above 30 μM. However, when lower concentrations (1–10 μM) were used, DIP inhibited singlet oxygen-mediated photohemolysis stimulated by other phototoxic compounds. In the present study, the above-mentioned differ-

ences in the production of ROS by activated PMNs were exploited to assess the antioxidant effects of DIP by chemiluminescent methods. While inhibitory effects have already been reported for DIP using various different activators by means of the cytochrome c reduction technique [11, 12], the originality of our study lies in the comparison of the scavenging effects of the drug on chemiluminescence generated by different ROS released intra- or extracellularly during activation of PMNs. This strategy can afford valuable information about the antioxidant properties of DIP because, in contrast to the spectrophotometric cytochrome c reduction technique, which only detects the extracellularly liberated $O_2^{\cdot-}$, chemiluminescence assays can differentiate between the metabolic stages of ROS such as the primary substrate level characterized by $O_2^{\cdot-}$ production (detectable by lucigenin-dependent chemiluminescence) and the MPO$-H_2O_2-Cl^-$ system-mediated level (detectable by luminol(s)-dependent chemiluminescence) [13].

2. Investigations and results

2.1. Effects of DIP on CL in PMNs activated by either PMA or fMLP

These results were obtained in five sets of experiments in which the interplay of different variables on the effects of DIP on CL in PMNs activated either by PMA or fMLP were investigated, and are illustrated in Figs. 1–5.

2.2. Effects of DIP on CL in PMNs activated by PMA (ILCL)

The results of this set of experiments are shown in Fig. 1. Dipyridamole in concentrations of 15, 25 and 50 μM inhibited the extracellular ILCL responses of PMNs activated by PMA in the presence of HRP in such a manner that light generation was observed after a lag period (Fig. 1A). The concentration dependence of the lag period is shown in the inset of Fig. 1A. Additionally, the traces after the lag period are parallel, suggesting that the cells were not affected by DIP. The extent of this lag-time did not increase significantly with the duration of preincubation of PMNs with dipyridamole at 37 °C. In fact, the mean lag-times in PMA-activated cells preincubated with 50 μM DIP for 0,5 and 30 min periods were 4.8 ± 1.0 ($n = 3$); 5.5 ± 1.3 ($n = 3$) and 4.3 ± 1.5 ($n = 3$) min respectively. On the other hand, when DIP (50 μM) was added after the stimulant, a lag period of 8.3 ± 2.4 min ($n = 3$) was observed (Fig. 1B). When cells were irradiated with visible light for 2 h in the presence of 50 μM DIP these lag periods were shortened from 5.5 ± 1.5 ($n = 3$) in non irradiated control cells to 2.5 ± 0.3 min ($n = 3$) suggesting that irradiation promotes decomposition of DIP.

In other experiments, when the ILCL responses of PMA-activated PMNs in the presence of 50 μM DIP were assayed in the absence of HRP, the lag period disappeared although there was a marked decrease ($43.6 \pm 8.1\%$ with

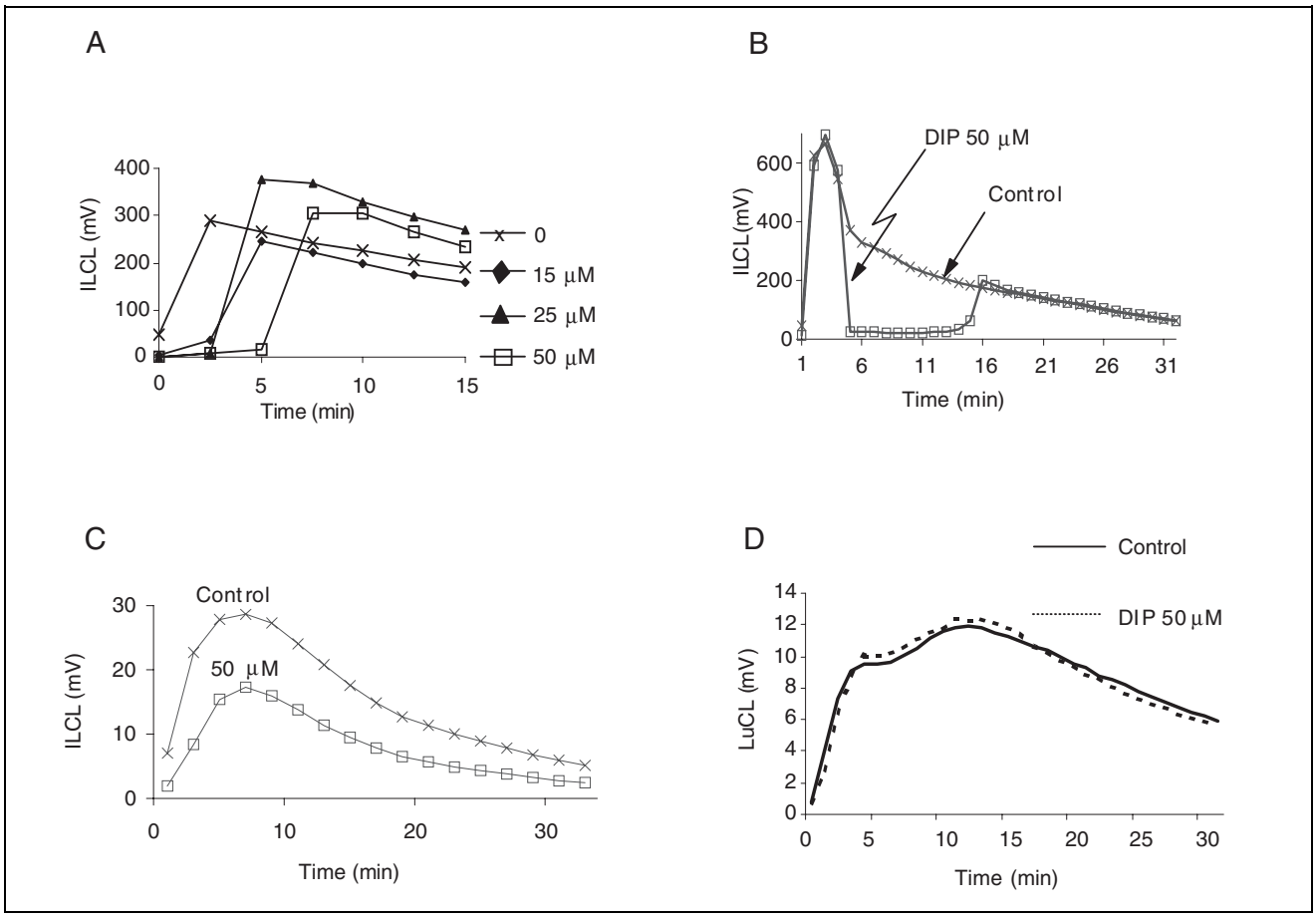


Fig. 1: Effects of dipyridamole on extracellular chemiluminescence. Panels A, B and C: Typical traces representing the effects of dipyridamole on PMA (0.22 μM)- induced isoluminol-enhanced chemiluminescence (ILCL) in human isolated PMNs (10^7 cells/mL). Dipyridamole at the concentrations indicated was added to PMNs either 5 min before (panel A) or 3 min after stimulant (panel B) in the presence of horseradish peroxidase (HRP, 3.9 U/mL). Inset in panel A, concentration-dependence of the inhibition period of chemiluminescence. Data at inset are shown as mean ± SD ($n = 4$). Panel C: Effect of 50 μM dipyridamole on time-course of ILCL of PMNs activated by PMA in the absence of HRP. Panel D: Effect of 50 μM dipyridamole on time-course of lucigenin (150 μM)-dependent chemiluminescence (LuCL) of PMNs (10^7 cells/mL) activated by PMA (0.22 μM).

respect to the control, n = 3) in ILCL response (Fig. 1C). Taken together, these results suggest that dipyridamole scavenges ROS generated by PMA-activated PMNs and that the existence of a lag period is a consequence of the presence of HRP. Furthermore, when evaluating the effects of dipyridamole (50 μM) on extracellular lucigenin-enhanced chemiluminescence in PMNs activated with PMA, no inhibition was detected (Fig. 1D) suggesting that dipyridamole does not scavenge the superoxide anion.

Extracellular lucigenin-enhanced chemiluminescence was not affected by 50 μM dipyridamole. On the other hand, 5 μM dipyridamole was sufficient to inhibit extracellular ILCL by more than 60% in formyl-methionyl-leucyl-phenylalanine (fMLP)-activated PMNs either in the absence or the presence of HRP.

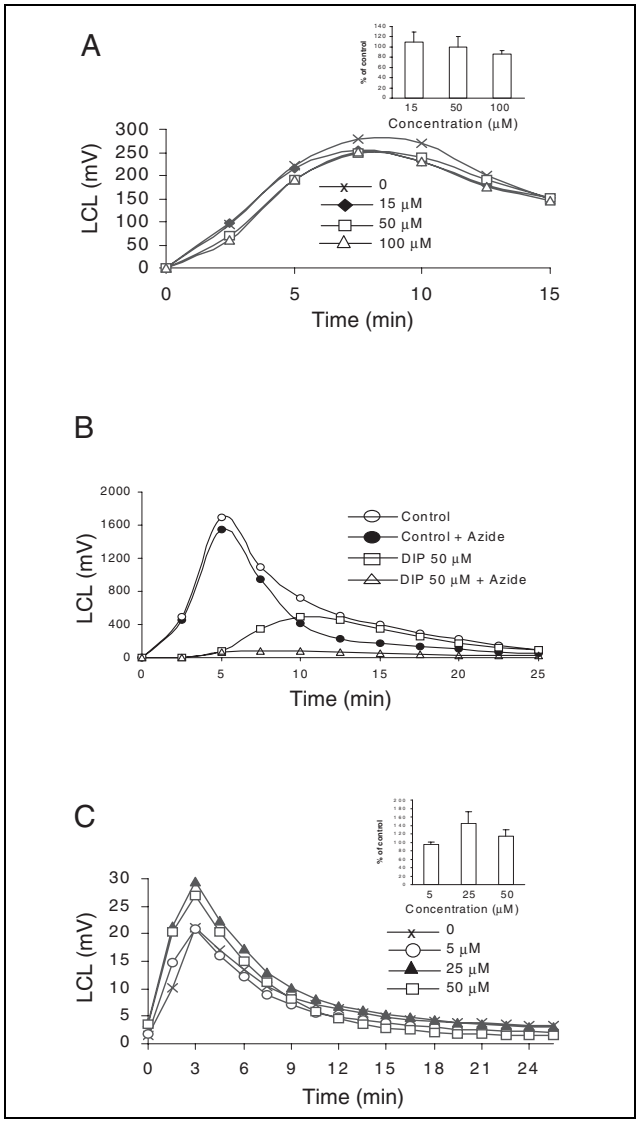


Fig. 2: Effects of dipyridamole on intracellular chemiluminescence. Panel A: Typical traces showing the effects of dipyridamole on PMA (0.22 μM)-induced luminol-enhanced chemiluminescence (LCL) in human isolated PMNs (10^7 cells/mL) in presence of superoxide dismutase (SOD, 20 U/mL) and catalase (2000 U/mL). Inset: Dose-dependent effect of dipyridamole on LCL. Data in the inset are shown as mean ± SD (n = 3). Panel B: Time trace of LCL emitted from human PMNs (10^7 cells/mL) when exposed to PMA (0.22 μM) without any additive (○) and in the presence of sodium azide (1 mM; ●), dipyridamole (50 μM; □) and dipyridamole + azide (Δ). Panel C: Typical traces showing the effects of dipyridamole on ionomycin (1 μM)-induced LCL in human isolated PMNs (10^7 cells/mL). Data in the inset are shown as mean ± SD (n = 3).

2.3. Effects of DIP in PMNs activated by PMA (LCL)

Luminol, in contrast to isoluminol, is able to penetrate into the cell [14], so that intracellular production of ROS on interaction with PMA in the presence of catalase and SOD can be monitored by a luminol-enhanced chemiluminescence technique (LCL). Therefore, when PMNs were compared with PMA (Fig. 2A) in the presence of dipyridamole (added 5 min before the stimulus) no significant inhibition was observed.

In order to rule out the involvement of some interaction between DIP and SOD or catalase, PMNs were stimulated by PMA in presence of HRP only. In this situation, the early phase of the LCL response reflects the extracellular production of ROS [15] (Fig. 2B) where the amount of secreted myeloperoxidase limits the measured activity. Because azide is a potent inhibitor of myeloperoxidase, the PMA-induced LCL reaction is abolished by the presence of azide unless an azide-insensitive peroxidase such as HRP is present in the medium. Therefore, upon addition of sodium azide, the LCL response is solely extracellular in origin, as in the system containing SOD and catalase. Again, addition of 50 μM DIP (in absence of azide) did not affect intracellular LCL response. Similar conclusions may be drawn by using ionomycin as the activating agent. This calcium ionophore activates PMNs in such a manner that production of reactive oxygen species (without added enzymes) is largely intracellular [16]. In this situation, the LCL response was only slightly increased (Fig. 2C). These results suggest that dipyridamole, at least in these short-term experiments, is unable to gain access to intracellular compartments.

2.4. Effects of Dip on CL in PMNs activated by fMLP

Incubation of PMNs with dipyridamole in the concentration range 0.1–5 μM, resulted in a dose-dependent loss in ILCL activity when the cells were activated by fMLP both in the presence (Fig. 3A) and in the absence (Fig. 3B) of HRP. In contrast with similar experiments using PMA-activated PMNs (Fig. 1A), ILCL response in PMNs activated by fMLP did not show the characteristic lag time between the initiation of the reaction and the beginning of light output, suggesting differences in the identity of ROS generated by either stimulus.

2.5. Reaction of DIP with hydrogen peroxide

The effects of the addition of H_2O_2 on the absorption spectrum of DIP are shown in Fig. 4. The band centered at 420 nm was not affected, suggesting that reaction of DIP with peroxide leads to very different products.

In the chemiluminescence experiments, DIP at concentrations of 50 and 100 μM inhibited the ILCL response by approximately 45 and 60%, respectively (Fig. 6). Hydrogen peroxide-induced ILCL with HRP was strongly inhibited by 2 μM DIP, and a nondose-dependent lag period was detected.

2.6. Reaction of DIP with hypochlorous acid

In cell-free systems hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$) and hypochlorous acid-induced ILCL responses were initiated by H_2O_2 (3.5 mM), $FeSO_4$ (40 nM) and NaOCl (100 μM) respectively.

HOCl is a powerful oxidizing and chlorinating agent generated by activated PMNs [17]. Since on the one hand,

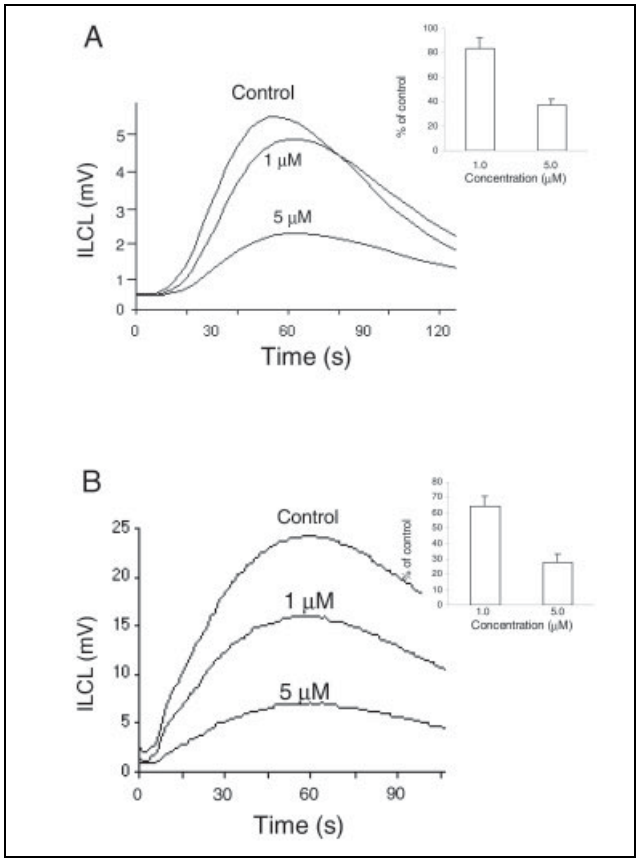


Fig. 3: Typical traces representing the effects of dipyridamole at the concentrations indicated on fMLP (0.57 μM)-induced isoluminol-enhanced chemiluminescence (ILCL) in human isolated PMNs (10^7 cells/mL) in absence (panel A) or presence (panel B) of HRP (3.9 U/mL). The insets in both panels show dose-dependent effects of dipyridamole. Data are shown as mean ± SD (n = 3).

DIP inhibited isoluminol-enhanced chemiluminescence in PMA-activated PMNs (Fig. 1C) and on the other hand, this cell-response presumably depends on HOCl release [7], we examined the possibility of a direct interaction between DIP and HOCl. Figure 7 shows that addition of HOCl caused marked changes in the absorption spectrum of DIP, which is indicative of formation of a new product. In particular, an apparent disappearance of the band centered at 420 nm suggests a loss of a π-conjugation system in the DIP molecule. Furthermore, this addition leads to a total loss of DIP fluorescence. Likewise, in the chemiluminescence assays a rapid response was induced by the addition of NaOCl to a buffer solution containing isoluminol (Fig. 6C). Dipyridamole at concentrations of 10 and 50 μM inhibited this ILCL response by approximately 20 and 80%, respectively (see inset in Fig. 6D).

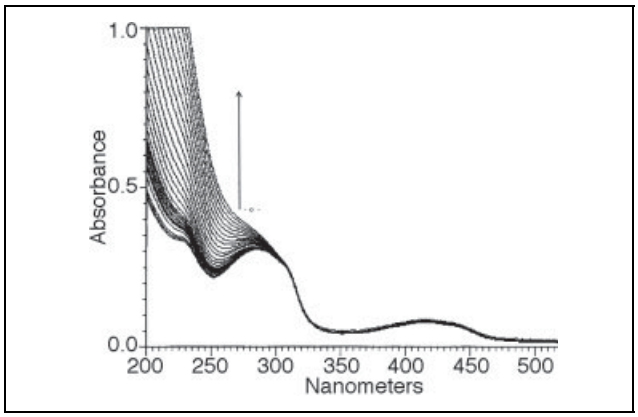


Fig. 4: Absorbance scans of DIP (1.0 μM) incubated with H_2O_2 (1.0 μM). The scans shown in this figure are about 30 s apart.

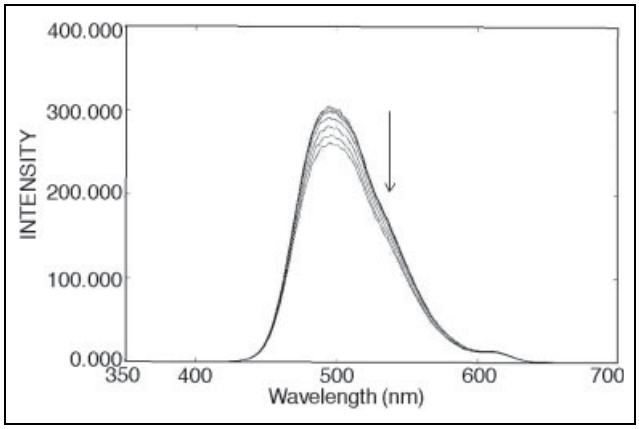


Fig. 5: Fluorescence scan of DIP incubated with H_2O_2 (λ_{exc} = 305 nm). The scans shown in this figure are about 30 s apart.

3. Discussion

The chemiluminescence data reported in this work suggest that dipyridamole is an efficient scavenger of $^{\bullet}OH$, H_2O_2 and HOCl. Although these antioxidant properties of dipyridamole have been postulated in previous papers [1, 2, 18], our findings reveal some other interesting properties of DIP in the presence of PMNs. For example, intracellular chemiluminescence was not affected by DIP (Fig. 2) probably reflecting the inability of DIP to penetrate into cells. On the other hand, lucigenin-dependent chemiluminescence was not inhibited by DIP, suggesting that superoxide anion ($O_2^{\bullet -}$) is not scavenged by the drug in the concentration range of 1–100 μM. Apparently, these latter findings contrast with those reported in previous works. In fact, as determined by both cytochrome c reduction [19] and electron spin resonance [20] techniques, DIP scavenged $O_2^{\bullet -}$ generated by a hypoxanthine-xanthine oxidase cell-free system. However, in the present investigation these scavenging effects could only be detected at higher concentrations of DIP (>100 μM). Furthermore, it is known that completely reduced xanthine oxidase produces both H_2O_2 and $O_2^{\bullet -}$ [21].

With respect to the different types of ROS generated by activated PMNs, it has been proposed that fMLP-stimulated LCL of PMNs predominantly reflects the generation of $^{\bullet}OH$ while PMA-stimulated LCL mostly reflects the formation of OCl^- and/or $^1O_2$ [7]. Although, the precise identities of ROS scavenged by DIP cannot be deduced solely on the basis of concentration dependencies, our results agree very well with these propositions because, in the first place, DIP inhibited both ILCL in PMNs stimulated with fMLP (Fig. 3) and ILCL in a cell free system initiated by ferrous sulphate (Fig. 6C), and on the other hand, the drug inhibited in a comparable manner ILCL in PMA-stimulated PMNs (Fig. 1C) and OCl^--stimulated ILCL (Fig. 6D). Furthermore, in a recent paper we proposed that DIP (1–10 μM) is a protector agent against singlet oxygen-mediated photohemolysis by phototoxic compounds, acting as a $^1O_2$ quenchive [10].

The results obtained from our chemiluminescence experiments carried out in presence of HRP are noteworthy, in that, although the origin of the ILCL (or LCL) response is not totally understood, the increase in light output is be-

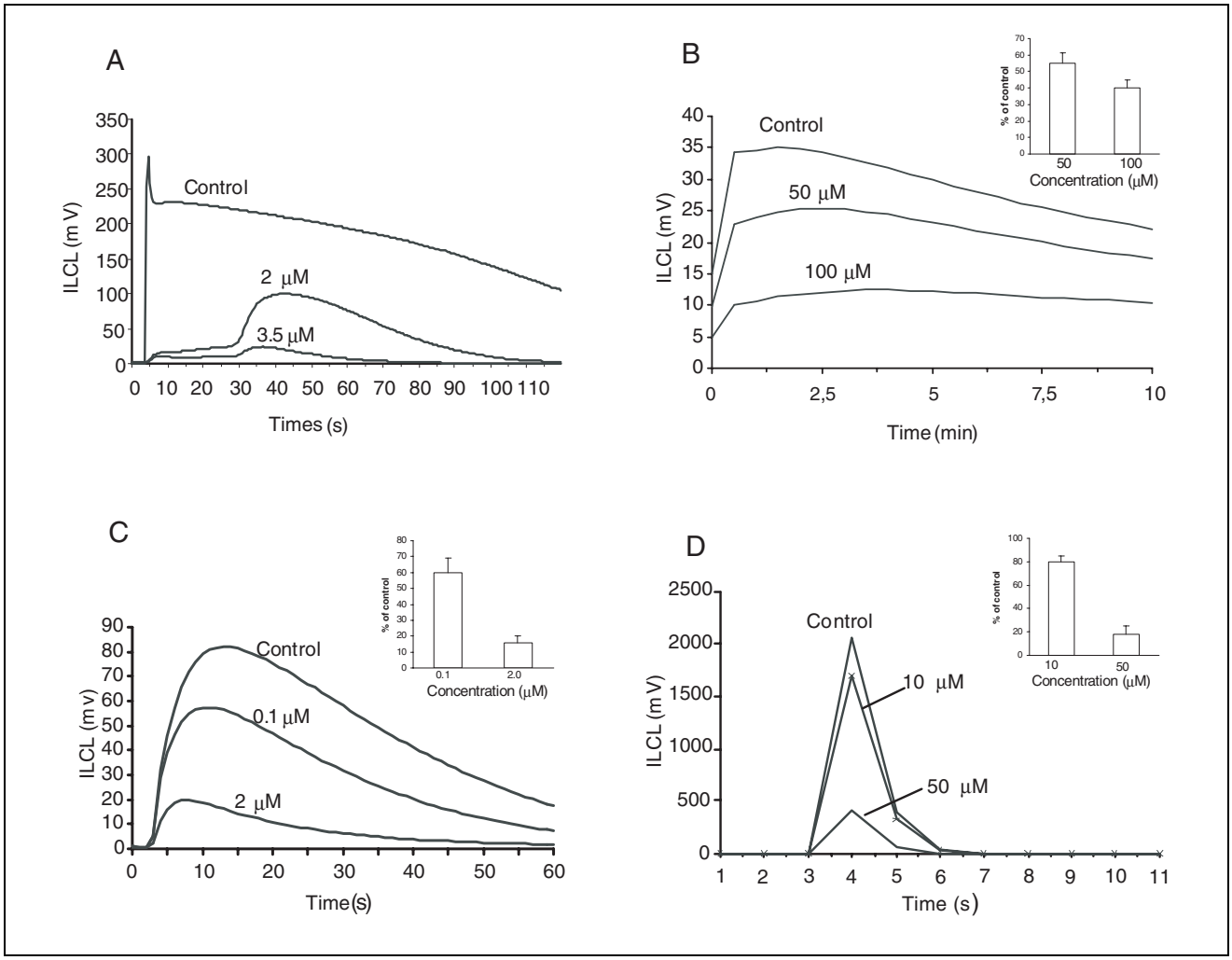


Fig. 6: Panels A, B, C and D: Effects of dipyridamole at the concentrations indicated on isoluminol-enhanced chemiluminescence (ILCL) in cell free systems. Figures show representative assays of the time course of ILCL after addition of: hydrogen peroxide (10 μM) to phosphate buffered saline, pH 7.4, with HRP (3.9 U/mL) and isoluminol (50 μM) (A); hydrogen peroxide to phosphate buffered saline, pH 7.4, containing isoluminol (250 μM, in 2 M NaOH) without HRP (B); ferrous sulphate (40 nM) to phosphate buffered saline, pH 7.4 (C) containing isoluminol (250 μM) (C); hypochlorite (50 μM) to phosphate buffered saline, pH 7.4 containing isoluminol (250 μM) (D). Insets in B and D show dose-dependent effects of dipyridamole. Data are shown as mean ± SD (n = 3)

lieved to come from reactive oxygen species that may oxidize the luminol to an excited state capable of producing chemiluminescence. For instance, luminol in a very basic solution reacts with H_2O_2 and generates LCL whereas at neutral pH, H_2O_2 will not interact with luminol unless a peroxidase is present. In both situations, DIP inhibited ILCL (Figs. 6A and 6B). However, when HRP was present, the chemiluminescence curves (Fig. 6A) showed a lag period which was not observed in its absence (Fig. 6B). This type of behavior was also observed in PMA-activated PMNs (see figs. 1A and 1C) suggesting that the lag period is a consequence of some type of interaction between DIP and radicals derived from interaction between H_2O_2 and HRP. In fact, it has been suggested that drug-derived radicals with reduction potentials lower than that of luminol destroy luminol radicals and inhibit chemiluminescence [22]. In our assays, the accumulation of isoluminol radicals could be inhibited by the presence of DIP in the reaction medium, thus giving rise to a lag time [23]. Alternatively, it is possible that the lag time phenomenon may involve a reversible alteration in HRP catalytic activity [24].

The differences in magnitudes of lag times observed in the cell free system (Fig. 6A) as compared to PMA-activated PMNs (Fig. 1A) could be a consequence of the surroundings, rather than ILCL being generated as a single pulse as in the cell free assay; PMNs-derived H_2O_2 is formed by a series of time-dependent processes of a transient nature, set in motion by PMA. As a further complication, in a recent report evidence was provided that HRP

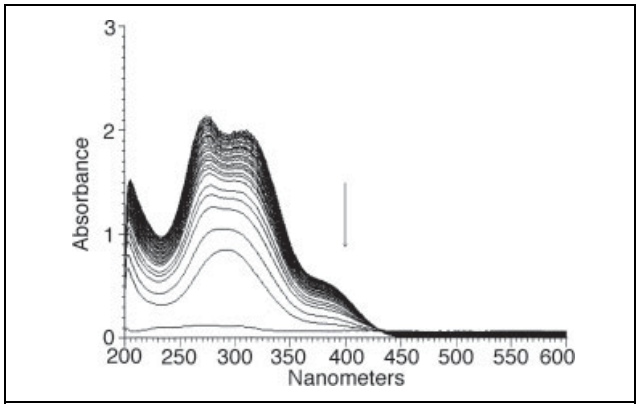
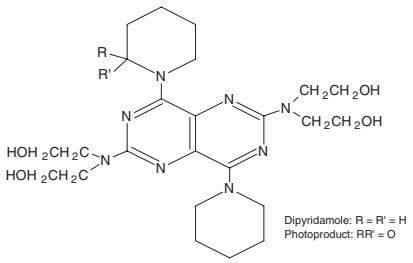


Fig. 7: Absorbance scans of DIP (1.0 μM) incubated with HOCl (1.0 μM). The scans shown in this figure are about 30 s apart

can catalyze a type of reaction that results in the production of $^{\bullet}OH$ from H_2O_2 in the presence of $O_2^{\bullet-}$ [25]. Independently of its origin, however, lag time measurements of peroxidase activities have been proposed as a reliable strategy for assessing the antioxidant activities of some compounds [23]. Interestingly, in a recent report it was shown that ascorbate induces a similar concentration-dependent lag period in the chemiluminescence of the cell-free system H_2O_2-luminol-HRP [26].

Peroxidases normally follow the reaction cycle: native enzyme → compound I → compound II → native enzyme, in which the last two steps involve hydrogen atom transfer from substrate to enzyme. However, rapidly reacting reducing substrates can transfer an electron rather than a hydrogen atom, resulting in the formation of a π-cation radical. In this connection, it was recently reported for the first time that DIP is oxidized in aqueous and micellar solutions by horseradish peroxidase-hydrogen peroxide forming a cation radical species [27]. In addition, the HRP-H_2O_2 system oxidized DIP such that its fluorescence was reduced almost to zero due to the non-fluorescent product formed upon oxidation. Our results indicate that both absorption and fluorescence spectra of DIP are altered when incubated with an equimolar concentration of H_2O_2 or HOCl in the absence of HRP (Figs. 4 and 5). In particular, on reaction with HOCl, the fluorescence of DIP was totally suppressed and the intensity of the absorption band around 420 nm was reduced almost to zero (Fig. 7). Only one photoproduct of such a reaction was detected and identified as the main product.



In agreement with this result, when PMNs were irradiated for 2 h in the presence of 50 μM DIP and subsequently stimulated by PMA, the lag period in ILCL response was clearly diminished from ∼6 min down to ∼2 min, suggesting that DIP decomposes on irradiation. This photodecomposition of DIP has been shown in a previous work [28] in which the authors demonstrated that on irradiation of a DIP solution in deuterated oxide, compared to normal water, an increase in the rate of photodecomposition of the drug was observed, suggesting that singlet oxygen might be involved in the photodecomposition mechanism. Furthermore, in a very recent work from our group it was found that irradiation of DIP under aerobic conditions leads to a loss of intensity of the 420 nm band as a consequence of a photodegradation process which probably occurs via a type II mechanism involving irreversible trapping of self-photogenerated singlet oxygen [29]. Therefore, oxidation of DIP by different ROS including singlet oxygen, is apparently accompanied by breaking the π-conjugation chain of the DIP molecule and by a concomitant total loss of scavenging properties [28, 30].

The dipyridamole molecule does not apparently contain any substituents in its structure that might be responsible for its scavenging action. For example, formation of chloramines upon reacting with OCl^- should not be considered excluded because dipyridamole lacks primary amino groups. However, it has been observed that some tertiary amines such as trimethylamine and the quinuclidine substituent of quinine react readily with HOCl, forming highly reactive chloro derivatives RN^+Cl^- [31]. It is tempting to speculate that upon reaction of DIP with OCl^-, the putatively formed quaternary chlorammonium ion could undergo spontaneous decomposition giving rise to a loss of fluorescence. On the other hand, it has been suggested that dipyridamole and H_2O_2 could react producing a cleavage of the piperidine ring with the formation of an aldehyde and with the subsequent formation of an N-oxide derivative [32]. Obviously, this proposal may be easily verified by characterization of the final oxidation product(s).

In conclusion, we have demonstrated that DIP, in the concentration range 1–50 μM, inhibits extracellular isoluminol-enhanced chemiluminescence in PMNs activated by either PMA or fMLP possibly by scavenging of $^{\bullet}OH$, HOCl and H_2O_2. In the same concentration range, intracellular chemiluminescence was not affected by the drug, suggesting that DIP is unable to penetrate into the cell. Due to these properties, we are currently evaluating the effects of other photosensitizing compounds on ROS-sensitive cellular and cell-free systems in the presence of dipyridamole.

4. Experimental

4.1. Chemicals

Dipyridamole, phorbol myristate acetate (PMA), formyl-leucyl-methionyl-phenylalanine (fMLP), ionomycin, luminol, isoluminol, lucigenin, horseradish peroxidase (HRP), hydrogen peroxide 30 wt.% solution in water and sodium hypochlorite (available chlorine ∼4%), superoxide dismutase (SOD) and catalase were purchased from Sigma (St Louis, USA). The HRP, SOD and catalase units are defined according to the Sigma catalogue. The concentration of HOCl was determined using an extinction coefficient of 100 M^{-1} cm^{-1} at 235 nm [33]. All the commercial reagents were used without further purification. Hank's balanced salt solution (HBSS) was prepared by dissolving the commercially available powdered medium (Sigma) in water. Before use, 0.1% (w/v) gelatin was added (HBSS-gel).

4.2. Cells

After informed consent, venous blood was obtained from healthy volunteer donors. Neutrophils were isolated from EDTA-anticoagulated blood following a differential centrifugation method as previously described [34]. The cells were separated and suspended to give 1.0×10^7 cells/mL of HBSS.

4.3. Neutrophil chemiluminescence

Chemiluminescence was measured in a Bio-Orbit 1251 luminometer using 4-mL polypropylene tubes with a 0.7 ml reaction mixture containing 10^7 cells/mL and also in a Luminoskan Ascent luminometer (ThermoLabsystems, Finland) in a 96-well Thermo Labsystems Microtiter plate. The tubes were equilibrated for 5 min at 37 °C after which the stimulus was added. The light emission was recorded continuously and its intensity was determined by integrating the area under the chemiluminescence curve during different times depending on the stimulant. Percentage chemiluminescence response at each dipyridamole concentration was expressed as the percent of the control value.

4.4. Luminol (LCL)-, isoluminol(ILCL)- and lucigenin (LuCL)- amplified chemiluminescence in activated PMNs

The chemiluminescence activity generated by isoluminol and HRP reflects the release of reactive oxygen species (ROS) from the cells, whereas the release of ROS as determined by luminol in combination with SOD and catalase reflects the intracellular activity [14]. The extracellular activity was detected using a mixture containing 56 μM isoluminol, 3.9 U/mL HRP. The intracellular activity was determined using a mixture containing SOD (20 U/mL) catalase (2000 U/mL) and luminol (20 μM). Dipyridamole was added at different concentrations or DMSO only (0.1%). The stimulants used to activate the cells were as follows: PMA (0.22 μM), fMLP (0.57 μM) and ionomycin (1 μM). Extracellular superoxide generation by neutrophils was measured using lucigenin-enhanced chemiluminescence (LuCL) as previously described [35].

4.5. Ferrous ion-induced chemiluminescence

Hydroxyl radicals were generated by the addition of freshly prepared $FeSO_4$ solution (40 nM, in 0.9% NaCl) to phosphate-buffered saline solution (PBS, 10 mM NaH_2PO_4 and 150 mM NaCl, pH 7.4). To this mixture,

luminol (250 μM) was added and CL was thereafter recorded continuously for 1 min [36]. The effects of various concentrations of dipyridamole were examined prior to the addition of the stimulant, $FeSO_4$.

4.6. H_2O_2-induced chemiluminescence

Hydrogen peroxide (3.5 mM) was injected into phosphate-buffered saline solution (PBS, 10 mM NaH_2PO_4 and 150 mM NaCl, pH 7.4), and then luminol (250 μM, in 2 M NaOH, diluted with PBS) was added and the mixture kept at 37 °C. CL was subsequently measured for 10 min [37].

4.7. HOCl-induced chemiluminescence

Hypochlorous acid/hypochlorite ($HOCl/OCl^-$,100 μM) was added to phosphate-buffered saline solution (PBS, 50 mM NaH_2PO_4 and 150 mM NaCl, pH 7.4) containing 20 μM luminol and different concentrations of dipyridamole. The luminescence was measured every second for 15 s [38].

4.8. Irradiation conditions

In some experiments the mixture of dipyridamole (50 μM) and PMNs (10^{-7} mL) was illuminated for 2 h with continuous stirring under aerobic conditions at room temperature. The light source used was a slide projector with a 300 W lamp (EXR, AV/Photolamp, Wiko, Japan). The irradiance was 50000 lux as measured by a Broad Range Lux/FC meter, Sper Scientific. Irradiated samples were assayed as described above and control experiments were carried out in the dark.

4.9. Monitoring of the reaction of dipyridamole with H_2O_2.and HOCl

Equimolar solutions (1 μM) of dipyridamole and H_2O_2 or HOCl were prepared and mixed in quartz cells. Repetitive spectra (200–550 nm) were measured every 30 s in a Milton Roy Spectronic 3000 diode-array spectrophotometer. In the same way, the reactions were monitored by spec\mittheta trofluorometry in a Shimadzu RF 1501 model spectrophotometer ($\lambda_{exc} = 305$ nm, $\lambda_{emis} = 480$ nm).

4.10. Statistical treatment of results

In each trial at least three independent experiments were performed unless stated otherwise. The figures show either single representative results or means (± SD where appropriate).

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