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Antioxidant activity of micronized diosmin on oxygen species from stimulated human neutrophils

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Abstract—Flavonoids are known to reduce reactive oxygen species released by polymorphonuclear neutrophils (PMNs) *in vitro*. We have studied the effects of S5682 (Daflon 500 mg), a purified flavonoid fraction composed of 90% diosmin and 10% hesperidin. S5682 produced a dose-dependent inhibition of the luminol chemiluminescence (CL) induced by phorbol myristate acetate on PMNs (IC_{50} = 5×10^{-5} M), with no effect on superoxide anion ($O_2^{\cdot-}$) formation and on cellular superoxide dismutase activity as determined by lucigenin-amplified CL. The CL results were confirmed by the hydrogen peroxide (H_2O_2) determination showing that S5682 reduced H_2O_2 formed through either PMN stimulation ($IC_{50} = 1.6 \times 10^{-6}$ M) or an *in vitro* enzymatic mechanism ($IC_{50} = 2 \times 10^{-6}$ M). S5682 inhibited luminol-dependent CL induced by H_2O_2 ($IC_{50} = 5 \times 10^{-6}$ M). However, O_2 was not formed from H_2O_2 in contact with S5682 and the UV spectrum of this compound was not modified. In contrast, S5682 inhibited luminol-dependent CL induced by H_2O_2 in the presence of horseradish peroxidase (IC_{50} = 3×10^{-6} M), and the UV spectrum of S5682 was modified. Luminol-dependent CL induced by hypochlorite (OCl^- 10^{-5} M) was also inhibited by S5682 ($IC_{50} = 7 \times 10^{-5}$ M). This inhibitory effect was similar to that of sodium azide on mycloperoxidase activity. Moreover, OCl^- 5×10^{-4} M also altered the UV spectrum of S5682 10^{-4} M. These results indicate that S5682 could be active on the H_2O_2–OCl^-–myeloperoxidase system.

Reactive oxygen metabolites are generated *in vitro* by polymorphonuclear neutrophils (PMNs*) during phagocytosis or after stimulation by phorbol myristate acetate (PMA) and other soluble agents. The respiratory burst occurring after PMN stimulation corresponds to an enhanced oxygen uptake. NADPH oxidase reduces oxygen [1], yielding superoxide anion ($O_2^{\cdot-}$) which is rapidly transformed into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) [2]. H_2O_2 can be then converted into hypochlorous acid (OHCl) by myeloperoxidase (MPO) [3] or can be a source of hydroxyl radical ($OH^{\cdot}$) in a Fe^{2+}-catalysed reaction [4]. Oxidative metabolites are involved in bactericidal processes and in tissue destruction occurring in the inflammatory reaction [5].

As flavonoids reduce respiratory burst [6], the effects of S5682 (Daflon 500 mg), a purified flavonoid fraction composed of 90% diosmin and 10% hesperidin, were studied. As this drug is poorly water soluble, it was solubilized by clathrate formation with β-hydroxycyclodextrin molecules. The effects of S5682 on PMN stimulation through reactive oxygen species formation were assessed by chemiluminescence (CL). Luminophores such as luminol (reacting with the whole set of reactive oxygen species) and lucigenin (more $O_2^{\cdot-}$ specific) were used to obtain highly sensitive light production [7–9]. Enzymatic and chemical techniques were also used to investigate the mechanism of action of S5682.

Materials and Methods

Isolation of PMNs. Blood samples were taken from healthy donors and collected in heparinized tubes. PMNs were separated by centrifugation (400 *g*, 20 min at 4°) on a Percoll gradient (72%/63%), 5 mL of total blood being deposited on the top. PMNs were clustered at the 72%/63% interface. After lysis of the erythrocytes, purity of the cell suspension was determined by MGG staining (95%) and cell viability of Trypan blue exclusion (>95%).

CL determination [9]. CL was measured in a LKB Wallac luminometer (model 1251) with two luminophores, luminol (aqueous solution containing 0.1% serum albumin) and lucigenin (pure aqueous solution). PMNs (3×10^5) in phosphate-buffered saline (PBS) containing luminophore 0.1 mM and the test compounds (10^{-4}–10^{-6} M S5682 or cyclodextrin as S5682 equivalent concentrations) were maintained in the luminometer for 5 min at 37° under agitation to obtain the background value. The stimulus compound PMA 10^{-7} M was then added and the luminescence measured every 120 sec after a 10-sec integration interval. CL was expressed in mV/3 × 10^5 cells. The effects of the test substances were determined at peaks of CL activity (maximum value from which the background value was subtracted) and IC_{50} values calculated from these peak values. SOD (100 U/mL), catalase (500 U/mL), NaN_3 (10^{-4} M) and flavonoids (10^{-4} and 10^{-5} M) such as hesperetin, quercetin and fisetin were also assessed. The effects of S5682 and cyclodextrin (10^{-5} and 10^{-6} M) on luminol-dependent luminescence produced by H_2O_2 (10^{-2} M) with or without horseradish peroxidase (HRP) (1 U/mL) and hypochlorite (OCl^-) (5×10^{-5} M) were also studied.

Spectrophotometric $O_2^{\cdot-}$ *determination.* (a) Cellular system: The kinetics of SOD-sensitive ferricytochrome *c* reduction by $O_2^{\cdot-}$ [10] were assessed in a suspension containing 10^6 PMN in PBS supplemented with NaN_3 (10^{-5} M). After a preincubation (5 min at 37°) of this mixture with ferricytochrome *c* (120 mM) and S5682 or cyclodextrine (10^{-4}–10^{-6} M), PMA (10^{-6} M) was added to trigger $O_2^{\cdot-}$ release. The reduction of cytochrome *c* by SOD-sensitive agents was determined after addition of SOD (30 μg). Ferricytochrome *c* reduction was determined at 5-min intervals, from 0 to 50 min, following addition of PMA, by measuring O.D. at 550 nm (Philips spectrophotometer FU 8720 UV/VIS) after centrifugation (400 *g* for 5 min). An exact value (ΔE) for the reduction of cytochrome *c* by $O_2^{\cdot-}$ was determined by subtracting the value obtained without SOD. The amount of $O_2^{\cdot-}$ released (nmol/10^6 cells) was determined by the following equation: ΔE 550 nm = 2.1×10^{-4} M^{-1} cm^{-1}. The effects of the

* Abbreviations: PMN, polymorphonuclear neutrophil; PMA, phorbol myristate acetate; CL, chemiluminescence; SOD, superoxide dismutase; MPO, myeloperoxidase; HRP, horseradish peroxidase; X, xanthin; XO, xanthin oxidase; G, glucose; GO, glucose oxidase; PBS, phosphate-buffered saline; PKC, protein kinase C.

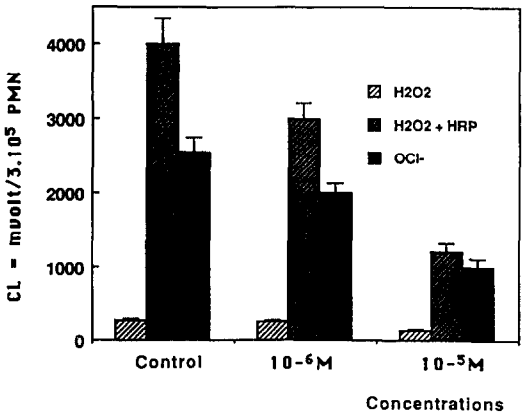

Fig. 1. Inhibitory effect of S5682 on luminol-dependent CL induced by H_2O_2 with or without HRP and by $OCl^{\cdot-}$. H_2O_2 (10^{-2} M final concentration) or $OCl^{\cdot-}$ (5×10^{-5} M final concentration) was added to luminol (0.1 mM), without or with different concentrations of S5682 in the absence or presence of HRP (1 U/mL). The CL peaks are expressed in mvolts. The results represent means ± SEM of triplicate determinations in three experiments.

inhibitors were investigated by comparing treated and non-treated cells and expressed as percentage response relative to control.

(b) Acellular xanthin/xanthin oxidase (X/XO) system [11]: The acellular system for $O_2^{\cdot-}$ generation was prepared with X (50 mM) ferricytochrome *c* (20 mM), NaN_3 (10 mM) and S5682 (10^{-4}–10^{-6} M). After a 5-min incubation, the reaction was initiated by XO (1.25 mU) and the amount of $O_2^{\cdot-}$ released determined at 20–25° as described above.

SOD activity of PMNs. SOD activity was determined by lucigenin-dependent CL [12]. SOD at different concentrations was evaluated by comparing its inhibitory effects on CL with those of SOD at known increased concentrations acting on the X/XO system.

After a 10-min preincubation with S5682 (10^{-4} M) and KCN (10^{-3} M) and a 30-min PMA stimulation (10^{-7} M), the ice-chilled cell suspension in PBS was sonicated (Branson Sonifier B30, 20 W) 10 times for 30 sec. X 2.5×10^{-5} M was added to start the reaction to 25 μL of the sonicate (10^6 cells) in a glycine-NaOH buffer containing lucigenin 0.1 mM and XO (4 mU/test). Luminescence was determined 30 sec after the onset of the reaction. Control samples containing PMNs were preincubated under the same conditions. The results were expressed in mU/10^7 cells.

Measurement of hydrogen peroxide release. Scopoletin oxidation was determined by spectrofluorometry [13]. Continuous fluorescence of reduced scopoletin was evaluated in a Perkin–Elmer spectrofluorimeter (MRF 31), with λex 390 nm and λem 460 nm.

(a) Cellular system: PMNs (3×10^5) were incubated (5 min, 37°) with S5682 and scopoletin (0.8 mM) in the presence of HRP (2.5 mM) and NaN_3 (0.1 mM) added to block extracellular H_2O_2 formation. PMA (10^{-6} M) was then added to trigger H_2O_2 release. H_2O_2 formation was expressed as the percentage of the fluorescence decay rate determined in the linear part of the curve.

(b) Acellular glucose/glucose oxidase (G/GO) system: For each concentration of S5682, GO (13 mU), scopoletin (0.8 μM) and HRP (2.5 μM) were added. After a 5-min preincubation at room temperature, fluorescence decay and H_2O_2 formation (mV/min) were determined as described for the cellular system.

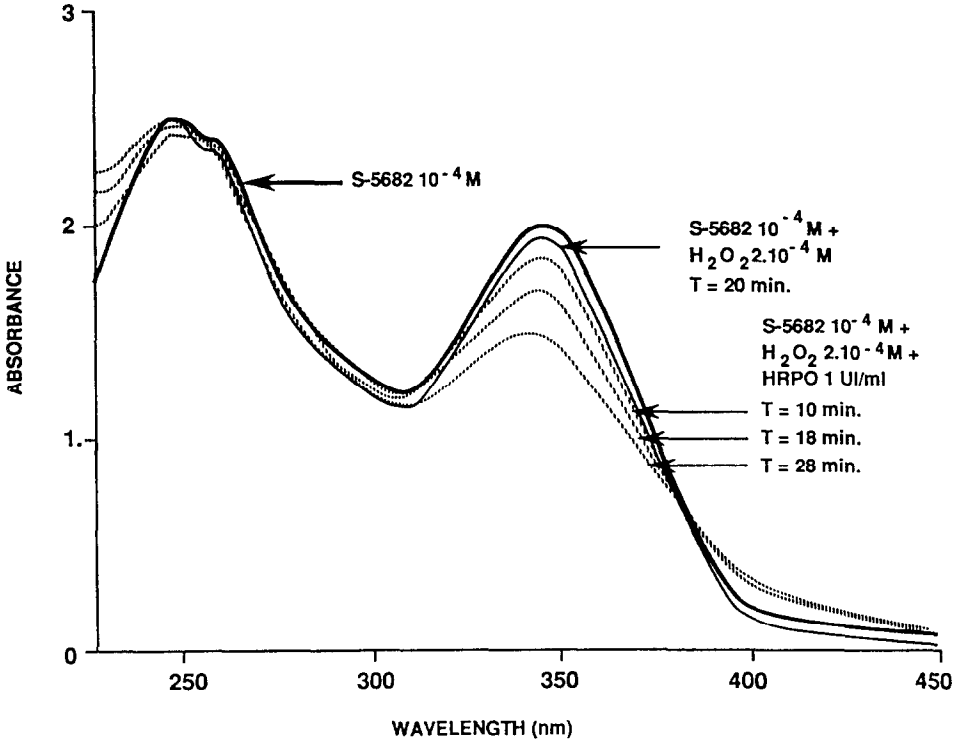

Fig. 2. Effect of H_2O_2 with or without HRP on the S5682 UV spectrum.

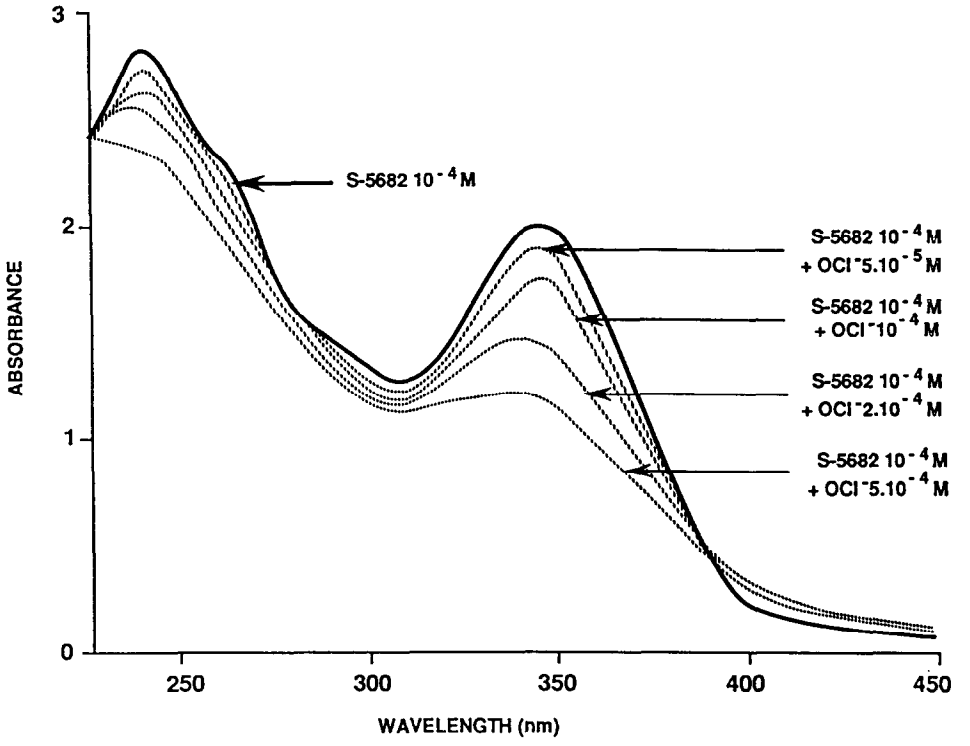

Fig. 3. Effect of OCl^- on the S5682 UV spectrum.

Measurement of O_2 release from H_2O_2 transformation. O_2 formation obtained by H_2O_2 breakdown was measured using a Clark oxygen electrode (Gilson Medical Electronics, K-IC Oxygraph). The test compounds were deoxygenated and injected into the vial in the following order: H_2O_2 10^{-4} M, S5682 10^{-5} M followed by 10^{-4} M. Catalase was tested at 10 U/mL. O_2 release was given in arbitrary units (lengths of time after addition of compounds).

UV spectrum analysis. The UV spectrum modifications of S5682 by H_2O_2 (10^{-4} M) were analysed in the absence or presence of HRP (1 U/mL). S5682 and cyclodextrin were tested at 10^{-4} M, in a Kontron UV-visible spectrophotometer. The spectrum was analysed every 2 min, for 20 min. UV spectrum modifications of S5682 promoted by OCl^- at 5×10^{-4}, 2×10^{-4}, 10^{-4} and 5×10^{-5} M were also studied.

Expression of results. All assays were run in triplicate and the results represent means from three different experiments. The inhibitory effect of the test compounds is given in percentage of control (100%). The Mann–Whitney U test was used for the statistical analysis.

Results

CL analysis. (a) Effects of S5682 on CL of PMA-stimulated PMNs: S5682 had no inhibitory effect on lucigenin-dependent CL. In contrast, a dose-dependent inhibition of luminol-dependent CL was observed with an IC_{50} of 5.10^{-5} M. Cyclodextrin did not inhibit CL. The comparison of S5682 with SOD, catalase and NaN_3 showed that NaN_3 and S5682 increased lucigenin-dependent CL (140 ± 10% and 120 ± 10%, respectively) but decreased luminol-dependent CL (14 ± 4% and 22 ± 5%, respectively, $P < 0.001$) and SOD decreased CL induced by both luminophores (12 ± 4% for lucigenin and 63 ± 8% for luminol). Catalase had an effect only on luminol-dependent CL (56 ± 10%). Quercetin and fisetin (10^{-5} and 10^{-4} M) inhibited CL induced by both luminophores with quercetin being more potent at 10^{-4} M (10 ± 5% and 3 ± 2% for lucigenin and luminol, respectively, $P < 0.001$). Hesperetin like S5682 increased lucigenin-dependent CL (126 ± 10 and 132 ± 20%, respectively) and inhibited luminol-dependent CL for both test concentrations (27% ± 5% and 6 ± 3%, respectively, $P < 0.001$).

(b) Effect of S5682 on SOD activity: S5682 (10^{-4} M) did not affect the SOD activity of PMNs, whereas KCN (10^{-3} M) decreased significantly (45 ± 5 mU and 13 ± 3 mU/10^7 PMNs, respectively, $P < 0.001$) this activity.

(c) Effect of S5682 on luminol-dependent CL induced by H_2O_2 or OCl^- (Fig. 1): A sharp response is induced by direct contact of H_2O_2 with luminol in the absence (within 4 sec) or presence (within 5 sec) of HRP and by direct injection of OCl^- (within 2 sec). S5682 inhibited, dose dependently, the luminol-dependent CL induced by H_2O_2 in the absence (IC_{50} 5×10^{-6} M) or presence of HRP (IC_{50} 3×10^{-6} M or by OCl^- (IC_{50} 3×10^{-6} M). Cyclodextrin had no effect and catalase inhibited totally CL induced by H_2O_2 (data not shown). These data showed that S5682 inhibited the luminescence induced by the oxygen species produced by H_2O_2 transformation.

Enzymatic and chemical technique analysis. (a) Effect of S5682 on $O_2^{\cdot-}$ release as assessed by spectrophotometry: S5682 had no effect on PMA-stimulated release by $O_2^{\cdot-}$ by PMNs. This was confirmed by the $O_2^{\cdot-}$ release observed in the X/XO system (data not shown).

(b) Effect of S5682 on H_2O_2 release determined by spectrofluorometry: PMA-stimulated PMNs released H_2O_2 which oxidizes scopoletin and abolishes fluorescence, proportionally to the amount of H_2O_2 released. When PMA-stimulated PMNs were preincubated for 10 min with increasing doses of S5682, the abolition of fluorescence was reduced (26% vs 15% and 9% for control vs S5682

10^{-6} and 10^{-5} M, respectively, and IC_{50} for S5682 1.6×10^{-6} M). The same effect was observed in the G/GO system, with an IC_{50} of 2×10^{-6} M. Cyclodextrin had no effect.

(c) Effect of S5682 on the transformation of H_2O_2: no O_2 formation occurred when S5682 (10^{-5} and 10^{-4} M) and H_2O_2 were directly in contact. The addition of catalase (10 U/mL) induced O_2 formation (+6 U of O_2 production).

(d) UV spectrum modifications: H_2O_2 did not modify the UV spectrum of S5682 even at 10^{-2} M (data not shown). The UV spectrum of S5682 was modified by addition of HRP with a decrease in the absorbance at 346 nm (Fig. 2). Moreover, the UV spectrum of S5682 (10^{-4} M) was modified dose dependently by OCl^- with a reduction of absorption at 340 nm (Fig. 3).

Discussion

In vitro stimulation of PMNs is followed by an emission of light (CL) which can be amplified by luminophores, such as luminol or lucigenin. In the first part of this study, we have shown that S5682 (90% diosmin and 10% hesperedin), reduced luminol-dependent CL triggered by PMA-stimulated PMN. Therefore, we have tried to localize S5682 targets and thus characterize the mechanisms of action of this compound.

Two enzymatic targets, protein kinase C (PKC) and SOD, could be crucial in characterizing S5682 activity. PKC is known to be essential in the activation of NADPH oxidase [14]. Flavonoids, such as quercetin, inhibit PKC activity [15, 16] and simultaneously $O_2^{\cdot-}$ formation. We have observed the same effect with fisetin and diosmetin, which inhibited lucigenin-dependent CL (strictly $O_2^{\cdot-}$ dependent) and could be active at this step of the cell response. Conversely, hesperetin and S5682 did not inhibit $O_2^{\cdot-}$ formation. Hence, the cascade of membranal events leading to NADPH oxidase activation was unaffected by S5682. SOD is essential in the transformation of $O_2^{\cdot-}$ into H_2O_2. KCN was shown previously to reduce SOD activity [17]. Under our experimental conditions where KCN (10^{-4} M) reduced SOD activity by 63%, S5682 had no effect on this enzymatic activity.

S5682 did not affect these two enzymatic targets. S5682 reduced the amount of H_2O_2 released after PMN induction and in the acellular G/GO system. Moreover, S5682 inhibited luminol-dependent CL induced by H_2O_2. This compound was more efficient than catalase and as efficient as NaN_3 in inhibiting luminol-dependent CL. The reaction was low, depending on the weak H_2O_2 artefactual transformation in the reaction mixture. Thus, under our experimental conditions, H_2O_2 alone did not modify the S5682 UV spectrum, and S5682 did not transform H_2O_2 into O_2. When HRP was added, the CL values were high as compared to the previous ones. S5682 inhibited under the same conditions (presence of HRP) the CL induced by H_2O_2 and its UV spectrum is modified. This suggests that S5682 may be active in the H_2O_2/OCl^-/myeloperoxidase system [7, 18]. Results obtained in the OCl^- study support this hypothesis. Indeed, S5682 inhibited luminol CL induced by OCl^- in an acellular system, and OCl^- induced a permanent modification of the S5682 UV spectrum. S5682-enhanced lucigenin-dependent CL might be explained by the inhibitory effects of this molecule on steps downstream to $O_2^{\cdot-}$ formation: the mechanism of this inhibition could be similar to that of NaN_3, a MPO inhibitor, which also increased $O_2^{\cdot-}$ formation. The enhancement of $O_2^{\cdot-}$CL might be explained by the greatly reduced utilization of this radical (through inhibition of MPO activity), or by NADPH oxidase stimulation, since OCl^- has a negative feedback effect on this enzymatic activity [19].

Our results demonstrate that S5682 affects in the same manner the product coming from H_2O_2 transformation and OCl^-, and may inhibit MPO activity.

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