With copper- and zinc-induced MT the half-lives of the protein are shorter and the metal and protein components of MT decline at the same rate[20,21]. For induction with zinc and the non-metallic inducers, the metal bound to MT is zinc. Reported values for the half-life of the protein component of zinc MT range from 9 h to 3 days[21-23,16-18] and a value of 1 day could probably be considered normal. When investigated, it has been found that the zinc status of the animal affects the half-life of zinc MT. Oh et al.[18] found little difference between the half-lives of hepatic and renal MTs in rats fed basic and zinc supplemented diets (3 days for hepatic and 2 days for renal), but the half-lives were considerably shorter in animals fed the supplemented diet and then returned to the basal diet (1.3 days for hepatic and 1.4 days for renal). Cain and Griffiths[23] reported values of 9.3 h and 14.7 h for the half-lives of rat hepatic MT I and MT II in the case of zinc injected rats and 16.4 and 26.1 h for the half-lives of the same MT iso forms from regenerating liver in non-injected rats. There is little published information on the activation energies for lysosomal protein degradation but if one assumed that the rate of protein degradation doubled for each 10 °C rise in temperature ($Q_{10} = 2$), then a half-life of rat MT of 1 day should correspond approximately to 7 days for the half-life of fish MT at 11 °C. The half-lives found in this study for fish MT are approximately 4 times this figure.

The results reported here indicate that zinc can produce enhanced levels of MT within a week, presumably by stimulating synthesis of fresh MT, and that this level remains high for at least 4 weeks at 11 °C. Fish MT levels could therefore represent a record of zinc exposure during the preceding weeks since we have not been able to show any effects due to stress. However, before conclusions can be drawn from MT levels in wild fish livers we need to know of any seasonal factors and of the influence on MT levels of dietary and water-borne metals.

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Ginkgo biloba extract inhibits oxygen species production generated by phorbol myristate acetate stimulated human leukocytes

J. Pincemail[a], A. Thirion[b], M. Dupuis[a], P. Braquet[c], K. Drieu[c] and C. Deby[a]

[a]Laboratoire de Biochimie et de Radiobiologie, Institut de Chimie, B6, Université de Liège, Sart-Tilman, B-4000 Liège 1 (Belgique), [b]Centre de Transfusion Sanguine de Liège, rue Dos Fanchon, B-4000 Liège (Belgique), and [c]IHB, IPSEN Institute for Therapeutic Research, F-92350 Le Plessis-Robinson (France), 2 December 1985

Summary. A Ginkgo biloba extract (Gbe) containing flavonoids, among other compounds, was tested for the release of activated oxygen species (O_2^-, H_2O_2, $OH^\cdot$) during the stimulation of human neutrophils (PMNs) by a soluble agonist. The extract slows down O_2 consumption (respiratory burst) of stimulated cells by its inhibitory action on NADPH-oxidase, the enzyme responsible for the reduction of O_2 to O_2^-. Consequently, superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) production is significantly decreased when the PMNs stimulation is done in the presence of the extract at concentrations of 500, 250 and 125 µg/ml. Moreover, the hydroxyl radical generation ($OH^\cdot$) is very much decreased at concentrations as low as 15.6 µg Gbe/ml, which indicates that the extract also has free radical scavenging activity. Gbe is able at least to reduce very severely the activity of myeloperoxidase contained in neutrophils. This enzyme, secreted into the intra and extracellular medium, catalyzes the oxidation of chloride (Cl^-) by H_2O_2 to yield strong oxidants (HOCl, chloramines) which are implicated in inflammatory processes.
Key words. Ginkgo; activated leukocytes; oxygen; radical scavenger.

The action of various stimuli (particulate or soluble agonists) on human neutrophils (PMNs) produces a marked increase of enzymatic oxygen consumption (respiratory burst) which results in reduction of O_2 to superoxide (O_2^-) via the NADPH-oxidase system[1,2]. O_2^- rapidly dismutates to yield hydrogen peroxide (H_2O_2); myeloperoxidase contained in neutrophils catalyzes the oxidation of chloride (Cl^-) by H_2O_2 to yield hypochlorous acid (HOCl)[3] and derivatives called chloramines characterized by nitrogen-chlorine (N-Cl) bond[4,5]. H_2O_2 can also generate the hydroxyl radical ($OH^\cdot$) in the presence of iron (Fe^{2+}). Lactoferrin secreted during activation of neutrophils seems to be a natural catalyzer of this reaction[6]. All these substances are toxic

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oxidizing agents and have bactericidal activities but they may also contribute to inhibition of neutrophil functions, tissue destruction and tumor promotion[7].

In this paper, we describe a study of the effects of a Ginkgo biloba extract[8] on the O_2 consumption and on the generation of three activated oxygen species (O_2^-, H_2O_2, $OH^\cdot$) during activation of human neutrophils by phorbol myristate acetate (PMA). The action of the extract on myeloperoxidase activity is also tested.

The Ginkgo biloba extract consists of two groups of major substances, flavonoid compounds and terpenoids[8]. In the first group, flavonoidic heterosides with quercetin and kaempferol as aglycone represent 24% of the extract. Besides these Ginkgoflavone glucosides, the extract also contains proanthocyanidins (24%).

In the second group, there are diterpenoids (ginkgolides) and a sesquiterpenoid (bilobalide) which are specific substances of the extract (6%).

Other substances of minor interest such as organic acids are present in the extract and play a role in its hydrosolubility. Ginkgo biloba extract is a drug which has been used for a long time in human therapy and is well known for its free radical scavenging activities[9].

Material and methods. Blood was obtained from healthy volunteers. After centrifugation (1000 × g, 25 min), the platelet rich plasma was removed and the neutrophil fraction (PMNs) was prepared by a 6% dextran-500 sedimentation, a centrifugation over Ficoll-Hypaque and a Tris-NH_4Cl (170–160 mM) treatment. The cells were suspended in a phosphate-buffered (pH 7.4) medium (PBS) without Ca^{2+} and Mg^{2+} containing 7.5 mM glucose. In all experiments, neutrophils were stimulated by phorbol myristate acetate (PMA, 8×10^{-8} M final) in the presence of Ca^{2+} (2 mM) and Mg^{2+} (0.5 mM) at 37 °C during 30 min. The water soluble Ginkgo biloba extract (Gbe) was a gift of the Institut Henri Beaufour (France). Ferricytochrome C and α-keto-γ-methiol-butyric acid were purchased from Sigma Chemical Co.

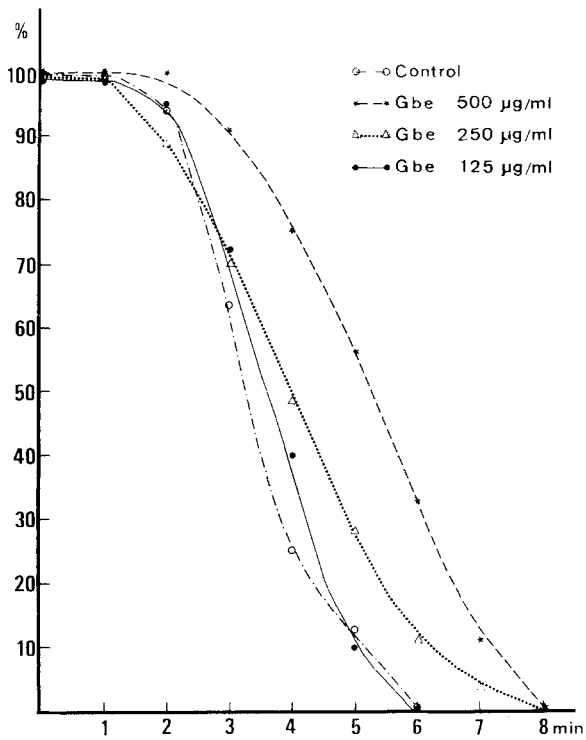

Figure 1. Effect of Gbe on O_2 uptake by PMA stimulated leukocytes. Abscissae: Time counted from the introduction of PMA in the oxygenated PMNs suspension. Ordinates: Decrease of oxygen concentration expressed as a percentage of its concentration (= 100%) before the PMA addition.

Table 1. Gbe inhibition of O_2^- production by PMA stimulated leukocytes. Values are mean ± SD (n = 3)

	nmoles O_2^-/10 min/1.5 10^6 cells
Control	28 ± 1.5
Gbe 500 µg/ml	18.75 ± 2
Gbe 250 µg/ml	20 ± 2
Gbe 125 µg/ml	24 ± 1.6
Gbe 62.5 µg/ml	28

Table 2. Inhibitory effect of Gbe on myeloperoxidase activity. Values are mean ± SD. Each experiment was made in duplicate

	% Inhibition
Control	0
Gbe 10 µg/ml	51.3 ± 1.0
Gbe 20 µg/ml	56.7 ± 0.9
Gbe 30 µg/ml	62.9 ± 0.7
Gbe 40 µg/ml	71.3 ± 0.2
Gbe 60 µg/ml	78.6 ± 2.4
Gbe 80 µg/ml	86.7 ± 1.2
Gbe 100 µg/ml	96.0 ± 0.7
Gbe 125 µg/ml	100.0

Cells viability. In the presence of the highest concentration of Ginkgo biloba extract used (500 µg/ml), the number of PMNs taking up the trypan blue dye (< 5%), did not increase, excluding an impairment of cell viability by the extract.

O_2 uptake. This uptake was monitored by a Clark-type oxygen electrode associated with a Beckman oxygenmeter and attached to a thermostatically controlled (37 °C) plastic vessel in which 2×10^7 cells/ml of PBS were stimulated by PMA in the absence (control) or in the presence of the extract[10].

O_2^- assay. The most common method of measuring superoxide anion is the ferricytochrome C assay. As Ginkgo biloba extract interfered with color development in this assay, PMNs were preincubated with the drug during 15 min. After centrifugation, the yellow supernatant was removed.

Cells were resuspended at a concentration of 1.5×10^6 cells/ml in PBS and stimulated by PMA. Superoxide-generating activity was measured by following the superoxide dismutase inhibitable reduction of ferricytochrome C at 550 nm in a double beam PYE-UNICAM spectrophotometer[11].

H_2O_2 determination. The amount of H_2O_2 released by 2×10^6 stimulated PMNs/ml after 30 min was determined according to the potassium thiocyanate method[12] in the presence of 10 mM azide in order to inhibit myeloperoxidase activity.

$OH^\cdot$ determination. 2.5×10^6 cells/ml were stimulated in the presence of 1 mM α-keto-γ-methiol butyric acid (KMB). The hydroxyl radical generated during the PMNs activation reacted with KMB to produce ethylene, measured by gas liquid chromatography, using a Porapak T column in a Barber-Colman 3000 gas chromatograph with FID[13].

Results were expressed as a % of the control value obtained by PMNs stimulation in the absence of Ginkgo biloba extract.

NADPH-oxidase activity. The 27,000 × g particulate preparation from neutrophils stimulated with phorbol myristate acetate was isolated as previously described[14]. The activity of the preparation was not measured by the NADPH oxidation (Ginkgo biloba extract strongly interfered at 340 nm) but by oxygen consumption using a Clark electrode as described above. The 27,000 × g pellet was resuspended in 0.34 M sucrose-Tris at a concentration of 1.3 mg protein/ml. The oxygen uptake was followed during 10 min after addition of 1 mM NADPH in the absence (control) or in the presence of Ginkgo biloba extract.

Myeloperoxidase assay. Myeloperoxidase with an absorbance ratio (A_{430}/A_{280}) of 0.7 was purified from human neutrophils according to Bakkenist et al.[15]. Enzyme activity was measured spectrophotometrically at 20 °C: 0.9 m U (4.4×10^{-8} M) of myeloperoxidase was combined with 2.9 ml of 50 mM phosphate buffer, pH 6.0 containing 0.617 mg/ml o-dianisidine hydrochlo-

ride (Sigma) and 1.4×10^{-4} M hydrogen peroxide[16]. The change in absorbance at 460 nm in the absence (control) and in the presence of the drug was measured during 1 min. Results are expressed in % of inhibition.

Results. O_2 Consumption. Gbe exhibits a significant effect on O_2 consumption of PMA stimulated neutrophils at 250 µg and 500 µg/ml (fig. 1): a 50% oxygen consumption is obtained after 4.1 and 5.2 min respectively, instead of 3 min when cells are stimulated without drug. However, the time for a complete oxygen uptake is the same for the two concentrations (8 min), while the same effect is obtained after 6 min with the control.

Fifty percent oxygen uptake due to the NADPH oxidase activity contained in the 27,000 × g pellet of human neutrophils is obtained after 1.5 min in the control. The presence of Gbe at 500, 250, 125 µg/ml delays this time until 4.0, 2.7 and 2.1 min, respectively (fig. 2). Moreover, the oxygen consumption in the presence of the extract does not reach 100% after 8 min (control) and is, for example, equal to 70% with Gbe at 500 µg/ml.

Superoxide anion production. Table 1 shows the results obtained with four concentrations of Gbe. With 500 µg/ml, there is a production of 18.75 ± 2 nmoles O_2^-/10 min/1.5 × 10^6 cells corresponding to an inhibition of 33%. At 250 and 125 µg/ml, the inhibitory effect is respectively of 28.5 and 15%, while at a much lower concentration (62.5 µg/ml), no effect is observed.

Hydrogen peroxide generation. The amount of H_2O_2 generated during PMNs activation by PMA can vary from 10 µM to 55 µM depending on the blood donor. The results expressed in percent of the control in figure 3 show that Gbe also inhibits the production of H_2O_2: there is an inhibition of 53 and 36% with 500 µg/ml and 250 µg/ml. For 125 and 62.5 µg/ml no significant effect is observed.

OH· generation. The ethylene production measured indicates that Gbe is a powerful hydroxyl radical scavenger. Indeed the extract is able to give an inhibition of 42% at a concentration as low as 15.6 µg/ml (fig. 4). At much higher concentrations, the inhibitory effect is more marked and can reach 85% at 250 µg/ml, for example.

Myeloperoxidase assay. Table 2 indicates that the inhibitory action of Gbe on myeloperoxidase activity is dose-dependent in the range of 10–100 µg/ml. At 125 µg Gbe/ml, the enzyme activity is completed inhibited.

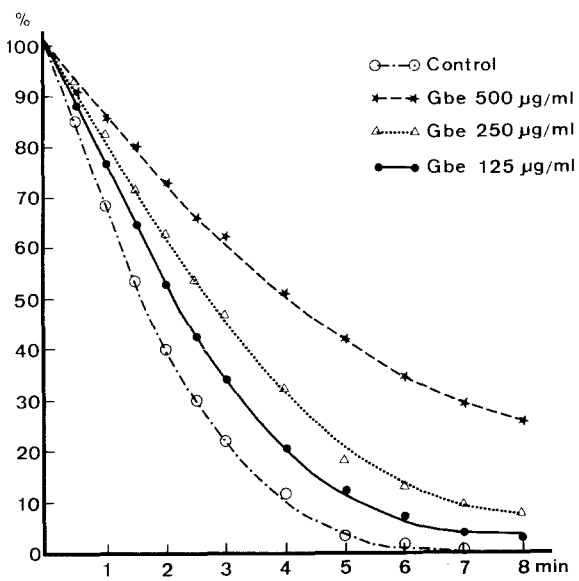

Figure 2. Inhibition effect of Gbe on NADPH-oxidase activity. Abscissae: Time counted from the introduction of NADPH in the 27,000 × g particulate preparation (1.3 mg protein/ml). Ordinates: Decrease of oxygen concentration expressed as a percentage of its concentration (= 100%) before the NADPH addition.

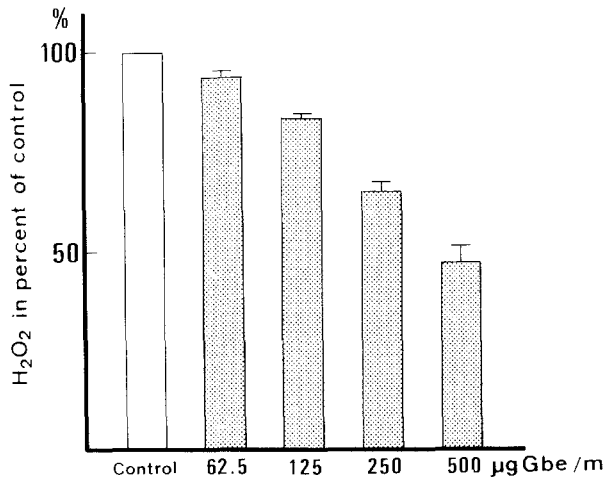

Figure 3. Gbe inhibition of H_2O_2 release from PMA-stimulated leukocytes. Values are mean ± SD (n = 4).

Discussion. The inhibition of the respiratory burst in preparations of whole PMNs, observed in the presence of Gbe, seems to be exerted at different levels.

1) Gbe inhibits NAPDH-oxidase activity, as shown in the broken-cell preparation. Tauber et al. have recently demonstrated that quercetin and kaempferol, two liposoluble flavonoids, were able to inhibit the respiratory burst enzymatic system[17]. Ginkgo biloba extract contains flavonoids such as kaempferol and quercetin heterosides which may be responsible for the extract activity.

2) As expected, the inhibitory effect of Gbe on NADPH-oxidase activity leads to a decrease in the release of superoxide anion. Yet it is surprising to establish that the inhibitory effect of Gbe at 500 µg/ml on hydrogen peroxide generation (54%) is more marked than that on superoxide anion (33%), although H_2O_2 arises from O_2^- dismutation. This difference can be explained by the fact that the two assays are not directly comparable. Indeed, the cells are preincubated with Gbe in the O_2^- assay and the reduction observed in O_2^- release thus results from the action of Gbe on NADPH-oxidase activity. For H_2O_2 assay, the stimulation was done in the presence of Gbe and consequently a direct O_2^- scavenging effect of the extract was possible, thus explaining why the inhibitory effect is higher in the H_2O_2 assay than in the O_2^- assay. In order to solve this problem, in another study, O_2^- was generated by an electrochemical pathway[19] and demonstrated either by the nitrobluetetrazolium (NBT) assay[18], or by the elec-

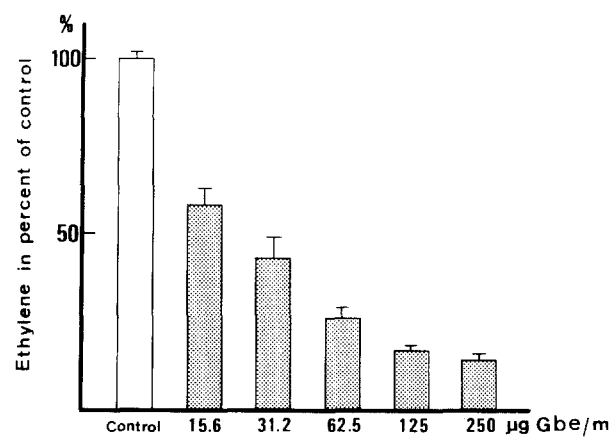

Figure 4. Hydroxyl radical scavenging effect of Gbe during PMA stimulation of leukocytes. Values are mean ± SD (n = 4).

tron spin resonance technic[19]. Unfortunately, Gbe interferes with color development in the first method and the freezing technic used in the second method gives unreproducible results so that it has not been proved in an indisputable way that Gbe scavenges O_2^-.

3) Gbe strongly scavenges $OH^\cdot$ generation in these experiments on PMNs. It is efficient at concentration as low as 15.6 µg/ml, while no effect is observed on O_2 uptake or on O_2^- and H_2O_2 release at this concentration.

This can be explained by the powerful free radical scavenging activity of Gbe, previously described in in vivo and in vitro experiments[20-22]. It easily reacts not only with $OH^\cdot$ but also with the 2,2-diphenylpicrylhydrazyl radical (DPPH) and the adriamycyl radical. Gbe also reduces in this way the free radical-induced lipoperoxidation generated by the NADPH-Fe^{3+} system in rat microsomes[9].

4) The myeloperoxidase activity which is responsible for the generation of strong oxidant species (HOCl, chloramines) is also significantly reduced by Gbe, even at low concentration (IC_{50} = 10 µg/ml).

Because of its regulator action on PMNs functions (inhibition of the NADPH-oxidase activity with, as a consequence, a decrease of release of activated oxygen species; scavenging of the hydroxyl radical, an oxidant species inducing lipoperoxidation phenomena; inhibition of myeloperoxidase activity), Gbe appears to be an interesting therapeutic agent. Indeed, our results give support to recent studies which have shown that PMA-induced superoxide anion release in polymorphonuclear cells from whole body gamma irradiated rabbits was significantly reduced in animals treated preventively with Gbe[23]. Besides its radiobiological protection, Gbe has been suggested for the reduction of the post radiotherapic edema in larynx cancer treatment, where there is an increase of O_2^- release by PMNs[24]. On the other hand, Gbe could be used experimentally in the treatment of diseases where free radical production by PMNs is suspected, as is the case in the Adult Respiratory Distress Syndrome (ARDS)[25,26] or in burn-injured patients[27].

Conclusion. Ginkgo biloba extract significantly reduces the production of oxygen species (O_2^-, H_2O_2 and $OH^\cdot$) during stimulation of human neutrophils by phorbol myristate acetate. Further studies are required to show the action of the extract on the chlorine derivatives mediated by myeloperoxidase and, on the other hand, to determine which fraction in the extract is the most active.

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Tetanus toxin does not affect the release of noradrenaline and taurine from rat cerebral cortex slices evoked by high K^+ and Na^+-free media

J. Heredero and S.S. Oja*

*Departamento de Bioquimica, Facultad de Medicina, Universidad Autonoma de Barcelona, Barcelona (Spain), and *Department of Biomedical Sciences, University of Tampere, Box 607, SF-33101 Tampere (Finland), 14 March 1986*

Summary. Noradrenaline and taurine release from superfused rat cerebral cortex slices was stimulated by potassium ions, veratrine, ouabain and omission of sodium ions. Tetanus toxin enhanced only the ouabain-evoked calcium-dependent noradrenaline release and the ouabain-evoked calcium-independent taurine release. The uptake of both was marginally affected.
Key words. Brain slices; noradrenaline; taurine; evoked release; uptake; tetanus toxin.

Tetanus toxin interferes with the release of several neurotransmitters from nerve endings[1]. Its main targets are the spinal inhibitory interneurons and their afferent terminals[2], in which the release of glycine and GABA is blocked. Consequently, the spinal motoneurons become hyperactive, giving rise to the spasticity and rigidity characteristic of manifest tetanus. In studies in vitro the type of tissue preparation used and the nature of the stimulus used to elicit the transmitter release are of impor-