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# Resveratrol and curcumin reduce the respiratory burst of *Chlamydia*-primed THP-1 cells

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**Abstract**

The intracellular bacterium *Chlamydia pneumoniae* is involved in the inflammation process of atherosclerosis. We previously demonstrated that *C. pneumonia* infected monocytes (THP-1 cells) responded to stimulation by an increased respiratory burst linked to an increased NADPH oxidase (NOX) activity. We now tested agents acting on the assembly of the NOX subunits or on protein kinase C, a trigger of NOX activity. Apocynin, resveratrol, rutin, quercetin, curcumin, and tocopherols were tested. The cells were pre-incubated with *Chlamydia* and the agent for 19 h, and then stimulated with phorbol myristate acetate. The NOX activity was monitored by measuring the hydrogen peroxide production. Resveratrol and curcumin ($10^{-4}$–$10^{-6}$ M) were better inhibitors than apocynin. α-Tocopherol was inactive, and γ-tocopherol inhibitor at $10^{-4}$ M only. Quercetin was inactive, and rutin a moderate but significant inhibitor. The inhibition by resveratrol was increased by $10^{-6}$ M rutin or quercetin. Resveratrol and curcumin thus appeared to be interesting for atherosclerosis treatment.


*Keywords:* THP-1; *Chlamydia pneumoniae*; Resveratrol; Curcumin; NADPH oxidase; Priming; IL-8; TNFα

Atherosclerosis, the main cause of morbidity and mortality in developed countries, is now considered as a chronic inflammatory disease. The primary lesion of atherosclerosis seems to come from excitations of the blood monocytes: these cells penetrate through the endothelium into the arterial intima, where they degenerate into specific cells, the *foam cells*, crammed with lipid droplets [1]. Before this final stage, monocytes are transformed into macrophages, large cells highly destructive for arterial tissues, thanks to their metalloproteinases and their capacities to generate reactive oxygen species (ROS) [2]; this transformation was explained by the ingestion of oxidized light density lipoproteins (LDL) [2]. But the reason for in vivo LDL oxidation remained unexplained. Macrophages exert chemotactic activities, attracting circulating monocytes, thanks to some cytokines and chemokines, such as IL-8, which is recognized to be implicated in atherosclerosis.

The primary cause of the monocyte appeal in determined points of the arterial net has remained unknown for a long time. Initiated by the epidemiological investigations of Saikku et al. [3], a series of studies showed in the following decade that an obligate intracellular bacterium, *Chlamydia pneumoniae* (*Chpn*), could be one of the irritant spines attracting the monocytes and leading more or less slowly to the atherosclerotic plaque.

In 1998, Kalayoglu and Byrne [4,5] showed that mouse monocytes, incubated with non-oxidized human

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LDL and *Chpn*, underwent conversion into macrophages, which became characteristic foam cells, the LDL being oxidized in the cells; inversely, incubation of non-infected monocytes with LDL did not produce foam cells.

In 2001, we obtained the same results as Kalayoglu et al., using a human monocyte line, THP-1, and fresh human LDL, in the presence of *Chpn* [6]. After triggering by phorbol myristate acetate (PMA), the infected monocytes–macrophages produced as twice oxidant species as non-infected monocytes. We demonstrated that the rise in oxidant activity was dependent on the *Chpn*-induction of NADPH oxidase (NOX), a widespread enzyme, which is present under different forms in leukocytes, endothelial cells, and vascular smooth muscle cells [7]. NOX is considered as the major source of ROS in the arterial walls [8]. Our observations were confirmed later by other groups [9].

*Chpn* infection constituted thus a priming for monocytes. Therefore, the hypothesis of a triggering role played by chlamydial infection in atherosclerosis was reinforced. It appeared more and more likely to us that a therapy aimed at lowering the activity of the NOX could slow down the development of the atherosclerotic process.

Using *Chpn*-infected THP-1 as model, and a specific technique for the measurement of hydrogen peroxide ($H_2O_2$), we began a series of experiments in order to moderate the respiratory burst of infected monocytes. In the first step, we observed that the classical inhibitor of NOX, diphenyliodonium, inhibited efficiently the ROS generation by THP-1 [6]. Then, because atherosclerosis is of an inflammatory nature, we used glucocorticoids [10] and observed that hydrocortisone at very low concentrations ($10^{-6}$–$10^{-8}$ M) enhanced the burst, while methylprednisolone was without effect, but increased the expression of $p^{22phox}$, a constituent of NOX [7]. We also observed that glucocorticoids decreased the cytokine production of *Chpn*-infected monocytes.

In this study, we report the data obtained with agents considered in the literature as able to act on NOX or on protein kinase C (PKC), the protein that triggers the assembly of the NOX constituents [11,12]. We compared the effects of apocynin, considered as a specific inhibitor of NOX, of resveratrol (RSV), suspected to be an inhibitor of the vascular NADPH oxidase [13,14], and curcumin, an inhibitor of the PKC response to PMA [15]. Because resveratrol was described as an apoptotic agent for THP-1 cells, we also studied the apoptotic effects of this compound in our model and experimental conditions.

Other compounds were tested: quercetin and rutin, considered as inhibitors of NOX [16], and α- and γ-tocopherols, as tocopherol can inhibit PKC and, by this way, can impair the NADPH oxidase assembly and reduce the respiratory burst [17,18].

## Materials and methods

*Reagents*. Reagents were dissolved in phosphate-buffered saline (PBS, 50 mM, pH 7.4). Analytical-grade salts, glycerol, ethanol, dimethylsulphoxide (DMSO), mercaptoethanol, and EDTA were from Merck (VWRI, Belgium). Phorbol 12-myristate 13-acetate (PMA), γ-keto-methylbutyric acid (KMB), glucose, horseradish peroxidase (HRP), bovine serum albumin, sodium pyruvate, L-glutamine, and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (Hepes) were from Sigma–Aldrich (Bornem, Belgium). The culture media RPMI and MEM, and fetal bovine serum were from Invitrogen (Merelbeke, Belgium), and antibiotics (gentamicin and streptomycin) were from Sigma. Resveratrol, curcumin, apocynin, bertrol, piceatannol, α- and γ-tocopherols, rhapontin, and *trans*-stilbene were from Sigma. Particular care was afforded to the quality of water, especially in the case of KMB experiments: ultrapure water was obtained at 18 MΩ cm (EasyPure Systems, Fisher Bioblock Scientific, Tournai, Belgium).

*Culture of the promonocytic THP-1 cells*. THP-1 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in Falcon flasks, with RPMI 1640 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml streptomycin, 0.25% glucose, 110 μg/ml sodium pyruvate, 36 μl/L mercaptoethanol, and 2.38 mg/ml Hepes, and maintained at 37 °C in a 5% $CO_2$ incubator. Prior to the assay, the culture medium was centrifuged at 1000*g* for 10 min, the supernatant was discarded, and the cells were counted and distributed for the assays (see below). The presence of mycoplasma was searched, using the MycoAlert Kit (Cambrex Bioscience, Rockland, USA).

*Preparation of the whole extract of C. pneumoniae culture*. Mycoplasma-free *C. pneumoniae* (TW-183) was a gift from Professor Orfila (Université d'Amiens, France). The bacteria were propagated in McCoy cells (purchased from BioWhittaker, Europe) maintained in MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 10 μl/ml gentamicin, as previously described [6]. Confluent McCoy cells were infected by centrifugation with $5 \times 10^4$ inclusion forming units (IFU)/ml and cultured at 37 ° C, in a 5% $CO_2$ incubator. After 92 h, the culture medium was discarded, the McCoy cells were split with glass beads in fresh culture medium; after centrifugation, the supernatant was collected and conserved in sucrose phosphate buffer. The whole *Chpn* extract was titred for infectivity [19], for endotoxin level (by quantitative end point chromogenic Limulus assay, Coatest, Chromogenix AB, Mölndal, Sweden), and stored at −70 °C. Whole *Chpn* extract was used on THP-1 cells on the basis of its endotoxin concentration (2 pg endotoxin for $1 \times 10^6$ cells) [6].

*Oxidation assays of KMB*. This technique has been extensively discussed in our previous papers [6,10]. The monocytes are excited by PMA $10^{-7}$ M in the presence of KMB and HRP. HRP converts $H_2O_2$ released by cells into ROS, in the extracellular medium. ROS react with KMB to produce ethylene. But we greatly improved the technique in order to avoid a stress-induced handling of the biological material, and particularly the trypsination of the macrophages. We used an original device, which is represented in Fig. 1.

THP-1 cells were placed in multiwell plates ($2 \times 10^6$ cells/well) and incubated for 19 h in the culture medium (RPMI) with an amount of whole *Chpn* extract equivalent to an endotoxin charge of 3.3 pg. After incubation, the multiwell plate was placed on an aluminium plate (bottom plate; 5 mm thickness), the cap of the multiwell plate being removed and replaced by a rubber foil covering the six wells. Another aluminium plate (top plate) of the same thickness, pierced by six holes (5.5 mm diameter) facing the six wells of the plate, was placed on the rubber foil and fixed to the bottom plate by four screws. By this way, it

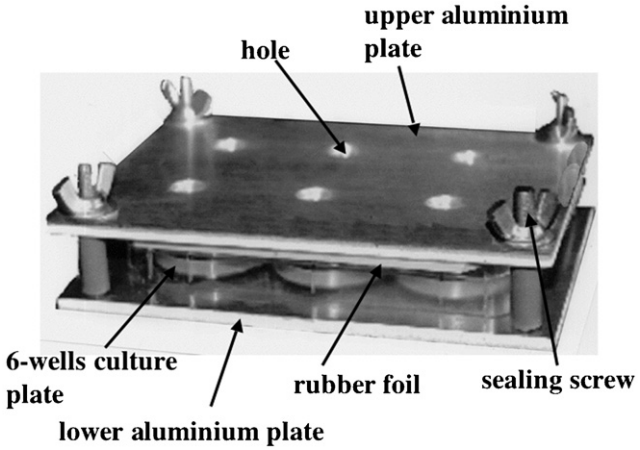


Fig. 1. Device used to measure the ethylene formation from KMB oxidation by the oxidant species produced by THP-1 cells under different experimental conditions.

was possible to incubate and test the cells directly in the multiwell plate, while avoiding trypsination and assuring that the individual cell compartments were sealed. The six holes of the top plate permitted us to inject the product and to take the gas sample to each well. KMB $10^{-3}$ M and the selected compounds were added to the wells before sealing. Two hundred units of HRP and $10^{-7}$ M PMA were added successively by plug puncture with a syringe (final volume: 2 ml) through the holes, and the devices were incubated at 37 °C for 2 h to allow the oxidation of the KMB molecule by the radical species, with production of ethylene.

At the end of the reaction, the level of KMB oxidation was estimated by measuring the ethylene amount released into the gaseous phase. Ethylene was quantified by gas chromatography on a Porapak T column (1 m length; ID 1/8 in; supplied by Supelco, Belgium) at 80 °C using nitrogen as vector gas (40 ml/min), with flame ionization detector at 120 °C. The gas chromatography column was standardized with pure ethylene ($C_2H_4$-25 quality, Air Liquide, Belgium). The gas samples were obtained by the use of a 1 ml Hamilton gas syringe A-2 (Vici Precision Sampling Inc.).

All the tested molecules, apocynin, RSV, curcumin, α- and γ-tocopherols, rutin, quercetin, *trans*-stilbene, bertrol, piceatannol, and rhapontin, were dissolved in ethanol at an initial concentration of $10^{-2}$ M and further diluted in PBS. Control assays with equivalent amounts of ethanol were performed on *Chpn*-infected monocytes.

*TNFα and IL-8 production*. Cells ($1 \times 10^5$ cells by assay) were incubated overnight with whole *Chpn* extract, with or without addition of the tested molecules. After incubation, the supernatants were collected, centrifuged to eliminate non-adherent cells, and frozen until the cytokines were measured by a sandwich enzyme immunoassay (Quantikine HS human TNFα and Quantikine human IL-8, R&D Systems, UK). The detection limits were 0.18 pg/ml for TNFα and 10 pg/ml for IL-8. Assays were repeated at least five times for the drug assays and 10 times for *Chpn*-primed THP-1 cells and for non-infected cells.

*Apoptosis measurements*. Cell apoptosis was detected with the Vibrant Apoptosis Assay Kit #1 (V-13240 from Molecular Probes). The kit included annexin V conjugated to the fluorophore Alexa Fluor 488 for apoptotic cell staining, and the Sytox Green dye impermeant to living and apoptotic cells but intensely staining necrotic cells. After staining, the apoptotic cells showed green fluorescence, the necrotic cells a very intense level of green fluorescence, and the living cells no or very little fluorescence. Stained cells were observed and counted with a fluorescence microscope (Axioskop, Zeiss, Germany) working with the 488 nm excitation and the 530 nm emission filters. For living cell count, a weakly transmitted light adjusted in phase contrast was used.

*Statistical analysis*. Mean values were given with the standard deviation (SD). Statistical difference analysis was performed using Student's *t* test, with $p < 0.05$ considered as significant (*), $p < 0.01$ as very significant (**), and $p < 0.0001$ as extremely significant (***).

## Results

### *Does RSV exert an apoptotic effect on THP-1 infected by Chpn?*

In each group, the assay was repeated six times, and four microscopic fields were counted for each well. The results are summarized in Table 1.

Non-infected THP-1 contained 14.18% of dying cells, and the addition of RSV enhanced the mortality, with a significant effect with RSV at $10^{-5}$ M (Table 1, group 3). But in *Chpn*-infected THP-1 (Table 1, group 5), the percentage of dying cells was strongly reduced and the incubation in the presence of RSV did not modify the data.

### *Effects of resveratrol, curcumin, and apocynin on the ROS production by infected THP-1 cells*

Fig. 2 shows the important and dose-dependent inhibitory effects exerted by RSV and curcumin on the oxidant production by *Chpn*-infected THP-1 cells; apocynin was less efficient.

Table 1
Percentages of dying cells (necrotic + apoptotic) in THP-1 populations treated for 19 h with RSV, in the presence or in the absence of *Chpn*

| | Non-infected THP-1 cells | | | | *Chpn*-infected THP-1 cells | | |
|---|---|---|---|---|---|---|---|
| Group number: | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Group conditions | No RSV | +RSV $10^{-4}$ M | +RSV $10^{-5}$ M | +RSV $10^{-6}$ M | No RSV | +RSV $10^{-5}$ M | +RSV $10^{-6}$ M |
| Mean | 14.18 | 18.85 | 20.40 | 22.53 | 4.03 | 3.5 | 1.7 |
| SD | ±2.65 | ±6.14 | ±3.89 | ±8.38 | ±2.45 | ±2.78 | ±2.05 |
| *p* value[a] | | 0.1183 | 0.0089** | 0.423 | 0.0001*** | 0.0001*** | 0.0001*** |

[a] The *p* values were obtained by comparison of the different experimental groups with the control group 1 (non-infected THP-1 cells).
** Very significant.
*** Extremely significant.

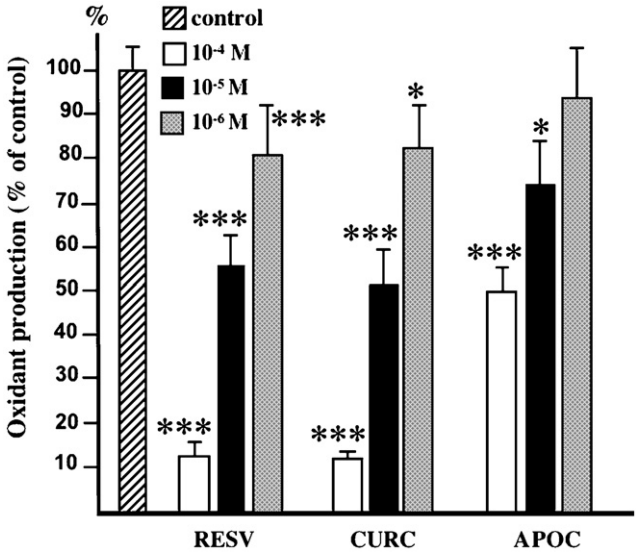


Fig. 2. Inhibitory effects of resveratrol (RESV), curcumin (CURC), and apocynin (APOC) on the ROS production by $2 \times 10^6$ THP-1 cells infected by *C. pneumoniae*. Incubation of cells with *Chpn* and reagents: 19 h. *p* values are given vs control: for RESV ($n = 15$) at all molarities, ***$p < 0.0001$; for CURC ($n = 8$) at $10^{-4}$ and $10^{-5}$ M, ***$p < 0.0001$, and at $10^{-6}$ M, *$p = 0{,}02$; for APOC ($n = 8$), at $10^{-4}$ M, ***$p < 0.0001$, at $10^{-5}$ M, *$p = 0.02$, and at $10^{-6}$ M, $p = 0.69$.

Table 2
Comparison of the effects of 5 stilbenoid molecules (at $10^{-5}$ M) on the ROS production by *Chpn*-infected THP-1 cells

| Experimental conditions | *n* | ROS production in % ± SD[a] | *p* value[b] |
|---|---|---|---|
| THP-1 + *Chpn* | 11 | 100 ± 11 | |
| THP-1 + *trans*-stilbene[c] | 6 | 39.5 ± 15.0 | 0.0023** |
| THP-1 + bertrol[c] | 3 | 27.7 ± 21.8 | 0.0008*** |
| THP-1 + piceatannol[c] | 4 | 60.9 ± 8.6 | 0.0253* |
| THP-1 + rhapontin[c] | 6 | 69.4 ± 8.7 | 0.0690 |
| THP-1 + resveratrol[c] | 6 | 42.0 ± 4.5 | 0.0025** |

[a] The results are expressed in percent of control (THP-1 + *Chpn*).
[b] The *p* values were calculated vs control (THP-1 + *Chpn*).
[c] The infected THP-1 cells were incubated 19 h with the stilbenoid molecule.
* Significant.
** Very significant.
*** Extremely significant.

### *Comparison of the effects of five stilbenoids on the ROS production by infected THP-1 cells*

The results are given in Table 2. The assays were performed at $10^{-5}$ M for each stilbenoid. Bertrol, RSV, and *trans*-stilbene were the most efficient agents, and rhapontin was the least inhibiting molecule.

### *Effects of several antioxidants on the ROS production by infected THP-1 cells*

Only γ-tocopherol at $10^{-4}$ M and rutin were very significant inhibitors of ROS production (Table 3). Inversely, α-tocopherol at $10^{-5}$ M enhanced the ROS production.

Table 3
Effects of rutin, quercetin, α-tocopherol, and γ-tocopherol on the ROS production by *Chpn*-infected THP-1 cells

| Experimental conditions | *n* | ROS production in % ± SD[a] | *p* value[b] |
|---|---|---|---|
| THP-1 + *Chpn* | 15 | 100 ± 7.6 | |
| THP-1 + rutin[c] | | | |
| $10^{-5}$ M | 6 | 79.1 ± 11.3 | 0.0003*** |
| $10^{-6}$ M | 6 | 85.2 ± 6.0 | <0.0001*** |
| THP-1 + quercetin[c] | | | |
| $10^{-5}$ M | 6 | 85.3 ± 25.0 | 0.0553 |
| $10^{-6}$ M | 12 | 90 ± 27 | 0.1996 |
| THP-1 + α-tocopherol[c] | | | |
| $10^{-5}$ M | 13 | 103 ± 29 | 0.7237 |
| $10^{-6}$ M | 15 | 118 ± 15 | 0.0056** |
| THP-1 + γ-tocopherol[c] | | | |
| $10^{-4}$ M | 11 | 46.9 ± 28.0 | <0.0001*** |
| $10^{-5}$ M | 10 | 102 ± 14 | 0.7061 |

[a] The results are expressed in percent of control (THP-1 + *Chpn*).
[b] The *p* values were calculated vs control (THP-1 + *Chpn*).
[c] The infected THP-1 cells were incubated 19 h with the molecule.
** Very significant.
*** Extremely significant.

### *Additional effects of RSV and flavonoids on the ROS production by infected THP-1 cells*

For RSV at $10^{-4}$ M, the additional effect of quercetin was without significant effect, but with rutin, the additional effect was very significant (Fig. 3). For RSV at $10^{-5}$ M, the additional effect of the two polyphenols

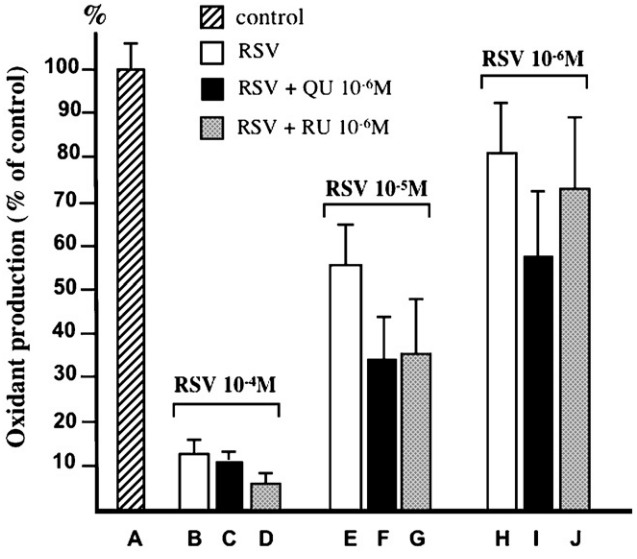


Fig. 3. Additional effects of polyphenols rutin (RU) or quercetin (QU) at $10^{-6}$ M on the inhibitory effect of resveratrol (RSV) on the ROS production by $2 \times 10^6$ THP-1 cells infected by *C. pneumoniae* (for each column, $n = 12$). *p* values: B vs C, $p = 0.3146$; B vs D, $p = 0.0006$; E vs F, $p = 0.0004$; E vs G, $p < 0.0001$; H vs I, $p = 0.0012$; H vs J, $p = 0.0975$; for A vs B, E or H, $p < 0.0001$.

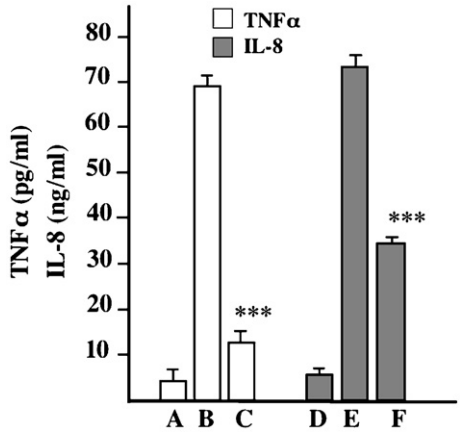


Fig. 4. Cytokine levels in the supernatant of $10^5$ THP-1 cells after 19 h incubation. (A,D) THP-1 without *Chpn* ($n = 10$); (B,E) THP-1 + *Chpn* ($n = 10$); (C,F) THP-1 + *Chpn* + RSV $10^{-5}$ M ($n = 5$). $p$ values: for B vs C, ***$p <$ 0.0001; for E vs F, ***$p < 0.0001$.

was very significant. For RSV at $10^{-6}$ M, only quercetin enhanced the inhibitory effect of RSV very significantly. At the three molarities, RSV alone inhibited the ROS production extremely significantly.

### *Effect of RSV on TNFα and IL-8 production by infected THP-1 cells*

The infection of monocytes by *Chpn* produced an important release of cytokines (Fig. 4). Resveratrol at $10^{-3}$ M decreased the cytokine production of *Chpn*-infected THP-1 cells in an extremely significantly way, particularly in the case of TNFα.

## Discussion

### *Importance of moderating NOX activity*

With our method, we measured the $H_2O_2$ released in the medium surrounding the excited monocytes, by using a peroxidase (HRP), which permitted the conversion of $H_2O_2$ into ROS. But what happens to the arterial tissues, in which normally no peroxidase exists? $H_2O_2$ is not active by itself and must be used by a peroxidase to produce ROS. This kind of peroxidase, the myeloperoxidase (MPO), exists in neutrophils. MPO uses hydrogen peroxide ($H_2O_2$) to generate hypochlorous acid (HOCl), a potent oxidant and an activator of pro-matrix metalloproteinase-7 in the arterial wall. MPO is thus indirectly responsible for LDL oxidation and plaque rupture in the atherosclerotic wall [20]. But MPO needs $H_2O_2$ to produce oxidant species, and $H_2O_2$ originates mainly from NOX activity [6,7].

The presence of MPO was detected in pathological arterial walls and its role in atherogenesis is now increasingly growing [21]. In 1995, our group demonstrated the uptake of MPO by endothelial cells [21]. Recently, Baldus et al. [22] demonstrated by immunohistochemistry the presence of MPO in the subendothelial compartment of sclerotic coronaries: MPO is a PMN-derived enzyme and could be sequestrated in vascular endothelial compartments. Myocardial ischemia and reperfusion recruit PMN, which release MPO; so, a diffuse endothelial distribution of MPO takes place [23]. Confirming this point of view, Gach et al. [24] observed that the stenting of an atherosclerotic coronary artery was followed, within a few minutes, by a dramatic peak of circulating MPO in blood. These findings confirmed the importance of the HOCl-producing MPO, but also the essential role of $H_2O_2$ generation. The key point for a pharmacological therapy of atherosclerosis is probably the regulation of the NOX enzyme.

### *Moderators of NOX activity and ROS production by infected THP-1 cells*

Our goal was thus to evidence new efficient moderators of the respiratory burst, which we monitored by measuring the amount of $H_2O_2$ released in the extracellular medium, and by this way, the potential toxicity of monocytes on the cell environment. The informations given by this technique of $H_2O_2$ measurement thus differ from those given by the techniques using intracellular redox dyes [25].

In a previous paper [6], we described the efficiency of diphenyliodonium (DPI), which, at $10^{-5}$ M, reduced to 40% the ROS released by stimulated infected THP-1 cells. But DPI now appears to be toxic [26,27]. The interest for finding NOX moderators other than DPI is thus justified. We took apocynin as a reference molecule to test the efficiency of two natural compounds, RSV chosen for its reputation to explain the ''French paradox'' [28], and curcumin. The French paradox consists in the low incidence of coronary artery disease in the French population, despite a high-fat diet. It was finally attributed to a high-wine consumption and to the presence in this beverage of flavonoids and mainly RSV [28].

The inhibition that we found with the tested molecules can be attributed to an activity on PKC, which is the trigger of NOX [15], or acts on the NOX component assembly [7]. Apocynin has been described as an effective inhibitor of PMN NADPH oxidase assembly and has recently been shown to inhibit the production of superoxide anion ($O_2^-$) by endothelial cells [29,30]. Resveratrol is considered to act on PKCa [31]. It decreased the NADPH oxidase activity in rat aortic homogenates [32], and, at 10–100 μM, the NADPH oxidase activity in macrophage homogenates [33]. Curcumin inhibited the fibroblast PKC [15] and the ROS generation by PMA-stimulated lymphocytes [34]. In our experiments, the popular apocynin appeared to be less efficient than resveratrol or curcumin.

*Does RSV exert an apoptotic effect on THP-1 cells infected by Chlamydia?*

Some contradictions appear in the literature about an induction of apoptosis in THP-1 cells by *Chpn*. According to Ojcius et al. [35], *Chlamydia psittaci* could enhance the secretion of IL-1β a sign that caspase-1, an apoptotic enzyme, was stimulated. Tsan et al. [36] published that RSV enhanced apoptosis in THP-1 cell line. On the other hand, another group reported that *Chpn* infection prevented natural apoptosis in THP-1 cells [37]. In our experiments, RSV increased the apoptosis of THP-1 cells, but this increase remained modest and was only significant at $10^{-5}$ M. It also clearly appeared that the *Chpn* infection reduced strongly the spontaneous apoptotic processes, as well as the apoptotic effect of RSV. These observations confirmed the validity of our model for RSV studies.

*Effects of other stilbenoids and some antioxidants on the ROS production by infected THP-1 cells*

Some stilbenes were as efficient, or even more efficient, than RSV. But this latter offers the advantage to have been better studied and to appear neither carcinogenic nor estrogenic. For the tested flavonoids, we found that rutin was active, but a lesser inhibitor than RSV and curcumin. Quercetin had no significant effect.

Cacchia et al. [38], using circulating human monocytes, observed that α-tocopherol inhibited the $O_2^-$ production by these cells after their stimulation by PMA, and explained these effects by an impairment of the NADPH oxidase constituent assembly. In our experiments, α-tocopherol was inactive, but we used *Chpn*-primed monocytes and detected the extracellular ROS, while Cacchia et al. monitored the intracellular redox state, using lucigenin luminescence; moreover, these authors incubated tocopherol only 30 min with the cells before the stimulation by PMA. The marked difference in behaviour between α-tocopherol and γ-tocopherol could be explained by an action of γ-tocopherol at the nuclear level, as observed recently by Li et al. [39].

*Additional effects of RSV and flavonoids on the ROS production by infected THP-1 cells*

From Table 3 and Fig. 3, it appears that the inhibiting effects of the flavonoids were additional. This additional action is interesting to explain the French paradox, as red wine is rich in both RSV and flavonoids [28]. However, a better synergy between flavonoid and resveratrol could perhaps be more apparent for incubation times longer than the 19 h used in our study [40]. New investigations are needed to better define the additional or synergic effects of RSV and flavonoids.

*Effect of RSV on cytokine production by infected THP-1 cells*

The infection of THP-1 cells by *Chpn* induced an important release of TNFα and IL-8 as previously described [10]. But RSV importantly reduced the release of these cytokines. TNFα is responsible for monocytes and PMN stimulation, and the pro-inflammatory cytokine IL-8 is one of the first chemokines to activate the PMNs after their priming with LPS; IL-8 is involved in PMN recruitment and activation [41]. The strong inhibition exerted by RSV on the release of these cytokines favours the view that this stilbenoid molecule is a good preventive agent against the inflammatory development of atherosclerosis.

## Conclusions

The NOX system is the main source of $H_2O_2$ (potential generator of ROS) during the monocyte excitation. Our results demonstrated that, to decrease the oxidant activity of NOX and the inflammatory cytokine production in monocytes infected by *Chpn* the most interesting of all the tested compounds were resveratrol and curcumin, already active at $10^{-6}$ M. The activity of RSV was further potentiated by a low concentration ($10^{-6}$ M) of the flavonoid rutin; moreover, RSV inhibited the inflammatory cytokine production in infected monocytes. These molecules could be interesting to limit or to inhibit the foam cell development, which is the first step in atherogenesis.

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