Potential anti-atherogenic cell action of the naturally occurring 4-*O*-methyl derivative of gallic acid on Ang II-treated macrophages

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Received 6 September 2004; revised 5 October 2004; accepted 5 October 2004

Available online 18 October 2004

Edited by Veli-Pekka Lehto

Abstract **We have recently established that the blood concentrations of gallic acid (GA), a polyphenolic component naturally found in food, and its *O*-methyl derivatives are very low (practically $\leqslant$ 1 μM) in physiological (postprandial) condition. Using acellular oxidant systems and macrophage-differentiated promonocytes (MDPs) THP-1, we show here that the direct and indirect (through depressing effect on the superoxide cell production) antioxidant properties of these components were not effective at these concentrations. In contrast, 4-*O*-methyl GA was the most efficient component to depress AT1R and CD36 mRNA expression in Ang II-treated MDPs, suggesting a strong inhibition of Ang II-triggered pro-atherogenic mechanisms of foam cell formation.**

Keywords: Low-density lipoprotein; Superoxide; Macrophage-differentiated THP-1; Ang II type 1 receptor; CD36

1. Introduction

The first stages of atherosclerosis leading to the formation of the lipid core (atheroma) imply endothelial cells, macrophage-differentiated monocytes and vascular smooth muscle cells (VSMC) of the vessel wall [1]. All these cell types are able to release bioactive substances, in particular reactive oxygen species (ROS) and cytokines, which play a crucial role in the pathogenesis [2]. It is worthwhile to notice that the production of a ROS, the superoxide anion ($O_2^{\cdot-}$), and the $O_2^{\cdot-}$-generating oxidase subunit expression are increased by several cytokines [3–5] resulting in the oxidized LDL (oxLDL) formation, whereas oxLDL is able to in turn stimulate $O_2^{\cdot-}$ production [6,7] and oxLDL receptor CD36 expression [8], leading to lipid accumulation and foam cell formation [9]. This could be considered as a self-amplification loop in which $O_2^{\cdot-}$-generating process and CD36 expression interfere.

Hypertension has long been considered as an atherogenic risk factor. The occurrence of a mechanistic link between hypertension and atherosclerosis is now established through the pleiotropic action of angiotensin II (Ang II) [10], the main peptide effector of the renin–angiotensin system. In addition to the vaso-constrictory action, Ang II is an inflammatory, chemoattractant, cell-growth and differentiation-promoting agent [11,12], and its production has been attested in the vascular wall [12,13], highlighting its pivotal role in atherogenesis. Ang II synthesis by macrophage-differentiated promonocyte (MDP) cells THP-1 has also been established [14]. Ang II action is mainly mediated by a cell-surface receptor, the Ang II type 1 receptor (AT1R) [15].

CD36, a class B scavenger receptor with multifunctional properties, is found in endothelial cells, monocytes/macrophages (and promonocyte cell line THP-1), VSMC, adipocytes and (cardio)myocytes. One of its functions is to recognize (minimally) oxLDL. Its activation leads to atherogenic and inflammatory processes [16]. It is abundantly represented in the core of the atherogenic plaque and generally considered atherogenic as strongly suggested by the CD36 null mice outcome [17]. It mediates enhanced H_2O_2 production in macrophages [18] (known to result predominantly from $O_2^{\cdot-}$-release) and its proper enhanced expression once activated by oxLDL [19].

Little is known on the role of Ang II in monocytes/macrophages. There is indirect evidence for an Ang II-induced increase in the respiratory burst and the oxidant stress [20–22]. It has been recently shown that Ang II-injected mice exhibited an increased expression of CD36 mRNA compared to non-injected mice [21].

Polyphenols (PP) – and particularly gallic acid (GA) – are antioxidant in vitro and protect other antioxidants in the biological fluids and LDL [23–26] in vivo. GA is found in plants and beverages in the free or bound forms [27]. 4-*O*-Methyl gallic acid (4-OMGA) has first been identified as a major methyl derivative of GA in human plasma and urine [28]. More recently, 4-OMGA and 3-*O*-methyl gallic acid (3-OMGA) have been found in urine during regular black tea ingestion [29] and in plasma after moderate red wine consumption [30].

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Abbreviations: AAPH, 2-2′-azobis-(2-amidinopropane)-hydrochloride; Ang II, angiotensin II; AT1R, Ang II type 1 receptor; CD, conjugated dienes; GA, gallic acid; 3-OMGA and 4-OMGA, 3-*O*-methyl gallic acid and 4-*O*-methyl gallic acid; MDP, macrophage-differentiated promonocyte; PMA, phorbol-12-myristate-13-acetate; PP, polyphenols; RDI, retinoic acid, vitamin D_3, interferon γ; SIN-1, 3-morpholinosydnonimine

0014-5793/$22.00
doi:10.1016/j.febslet.2004.10.015

The aim of this paper was to first compare the capacities of GA, 3-OMGA and 4-OMGA at protecting LDL against in vitro generated oxidative modifications, with a special reference to their physiological concentrations [30], and second to compare these antioxidant capacities to their cell-modulating action in Ang II-treated, differentiated THP-1, with a special reference to their action on $O_2^{\cdot-}$-production, on the one hand, and AT1R and CD36 mRNA expression, on the other.

2. Materials and methods

2.1. Materials

Gallic acid, angiotensin II, phorbol-12-myristate-13-acetate (PMA), 10,10′-dimethyl-9,9′-bisacridinium dinitrate (lucigenin), INFγ, and retinoic acid were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France), 2-2′-azobis-(2-amidinopropane)-hydrochloride (AAPH) was from Biovalley (Conches, France), 3-morpholinosydnonimine (SIN-1) was from Interchim (Montluçon, France). Standards of the *O*-methyl ether forms (3-OMGA and 4-OMGA) of GA were prepared from GA methyl ester according to Raju et al. [31]. Cell culture medium and fetal calf serum (FCS) were from Bio Media (France). 1α,25-dihydroxycholecalciferol was a gift from Hoffmann-La Roche SA (Basel, Switzerland). The ATP bioluminescent reagent (Vialight HS kit) was obtained from Cambrex Ltd (France). RNeasy mini extraction kit was from Qiagen (Courtaboeuf, France). Hybond N-nylon membrane was obtained from Amersham Biosciences Europe (Orsay, France).

2.2. Preparation, biochemical parameters and oxidizability of LDL

Human plasma was collected from normo-lipidemic patients undergoing plasmapheresis sessions in the Hematology Department of Hospital Lapeyronie (Montpellier, France). Preparation and determination of biochemical parameters of LDL were carried out as previously described [32,33]. LDL oxidation was performed at conventional (diluted) concentration already used [23,33] or at near physiological (more concentrated) concentration ($\leqslant$0.1 and 1 μM LDL, respectively, expressed as apo B) after a preincubation time period. For oxidation at conventional concentration, LDL (1 μM) was preincubated for 1 h at 37 °C under N_2 in the presence of products to be tested, and oxidized either by 5 μM Cu^{2+} after 10-fold dilution in PBS, or by 2.5 mM AAPH after 20-fold dilution, or by 21 μM SIN-1 after 10-fold dilution in PBS containing 10 μM DTPA. For oxidation at near physiological concentration, LDL was preincubated as above and oxidized without dilution in the presence of either 100 μM Cu^{2+} or 5 mM AAPH or 230 μM SIN-1. These concentrations were justified by the need to keep on a high oxidant/LDL molar ratio in order to obtain the same kinetics of oxidation – i.e., of conjugated diene (CD) production – as in the conventional condition [34]. LDL oxidizability was assessed by measuring the CD produced with monitoring at 234 nm in the case of Cu^{2+} and SIN-1 and at 245 nm in the case of AAPH [33]. Oxidation lag time was assessed for Cu^{2+} and AAPH, whereas CD production was directly measured for SIN-1. Respective mechanisms of action of the three oxidation systems have already been compared [33]. Briefly, the Cu^{2+}-oxidation system leads to Cu^{2+} interaction with an LDL low-affinity binding site (in the present condition of at least 50/1 Cu/LDL molar ratio) [34], then to the reduced form Cu^{+} which in turn leads to lipid peroxide formation. AAPH is able to directly produce radicals in the aqueous phase and thus to trigger lipid peroxidation independently of transition metals. The SIN-1, $ONOO^-$-generating system corresponds to a di-electronic process, in which $ONOO^-$ formation results from the simultaneous release of nitric oxide and superoxide by the 3-morpholinosydnonimine molecule, and the subsequent immediate reaction between them. This mimicks their simultaneous massive productions occurring particularly in the endothelial dysfunction involved in atherogenic processes.

2.3. Cell line and culture

The human THP-1 cell line was a gift from Dr. J. Dornan (University of Montpellier). Cells were maintained in culture and differentiated as previously described [33]. Briefly, for differentiation into adherent macrophages, promonocytes were plated in 96-well microplates (2.5×10^5cell/well of 0.2 ml) or seeded into a T-75 culture flask for 72 h in the presence of 1 μM retinoic acid, 0.1 μM vitamin D_3 and 100 U/ml IFNγ (the RDI mixture). This differentiating mixture was used instead of PMA in order to avoid the downregulating cell effect of prolonged PMA-incubation time on PKC and PKC-dependent events (including superoxide production) [35]. Then, the culture medium was replaced by the same one without the differentiating agents and MDPs were incubated further at 37 °C for 24 h with the substances to be tested. MDPs (100 μl) were incubated in the presence of 0.08% trypan blue for 5 min, and colored (dead) cells were observed under light microscope. The percentage of viable cells was superior to 95%.

2.4. Superoxide anion production assessment

The production of $O_2^{\cdot-}$ by MDPs was measured at 37 °C with lucigenin-amplified chemiluminescence (10^{-4}M lucigenin) by means of a microplate luminometer (EGG Berthold, France). MDPs were placed in the RPMI medium and incubated with Ang II for 24 h, in the presence or absence of PP. Finally, the medium was changed and PMA (0.1 μM) was added. The luminescence signal was counted over the 90-min period following PMA addition. In the control conditions, RPMI medium only contained the solvent vehicle used for PP dilution (ethanol, not exceeding 0.2% in the medium). The cell number was then determined by using a commercial kit for ATP extraction and determination. Briefly, this assay consists in the firefly luciferase-catalyzed oxidation of D-luciferin in the presence of ATP and oxygen, providing a direct relationship between ATP and the light produced. Results were expressed as the total amount of the $O_2^{\cdot-}$-generated luminescence per mol of ATP.

2.5. RNA isolation and Northern blot analysis

Total RNA was prepared from THP-1 cells using RNeasy mini extraction kit. Once differentiated and co-incubated in the presence of Ang II and PP to be tested, culture medium was aspirated, cells were washed twice with cold PBS (pH 7.4) and scraped. Cell suspension was then centrifuged (10 min, 2000 rpm) and submitted to RNA extraction. Total RNA (20 μg) was separated by 2% agarose/formaldehyde gel electrophoresis and transferred to a Hybond N-nylon membrane in 0.05 M NaOH. Northern blots were prehybridized for 1 h at 42 °C in the hybridization buffer and then hybridized overnight with a random-primed, [α-^{32}P]dCTP-labeled, CD36 or AT1R cDNA probe. Specific mRNA expression was quantified by means of a Fujix BAS1000 PhosphorImager. The mRNA for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used to normalize gene expression. cDNA was obtained by RT-PCR of total RNA (1 μg) and then used as a template for subsequent PCR. 5′-ATGTAACCCAGGACGCTGAG and 5′-AAGGCCTTGGATGGAAGAAC were used as sense and antisense oligonucleotides, respectively, in the case of CD36 primers and 5′-CTGGGGCTGCATTGTATTCT and 5′-AAACCAGTGTGGACCAGGAG as sense and antisense oligonucleotides, respectively, in the case of AT1R primers. The PCR products were purified and quantified on a 1% agarose gel electrophoresis.

2.6. Statistics

ANOVA and non-paired Student's *t* test were used for statistical analyses. Results were given as means ± S.D.

3. Results

Fig. 1 shows the IC_{50} values obtained for GA, 3-OMGA and 4-OMGA at conventional LDL concentration (Fig. 1A) and at near physiological LDL concentration (Fig. 1B). It appears clearly that in conventional condition, 4-OMGA was practically without protective effect or the less antioxidant form towards the three oxidation systems, whereas 3-OMGA was the most potent of the three forms, with the most protective effect exerted in the presence of the SIN-1 oxidation system. Interestingly, the results in Fig. 1B were different from those in Fig. 1A, since in the more physiological condition the most protective effect was found with 3-OMGA in the presence of the Cu^{2+} oxidation system. Taken together, Figs. 1A and B

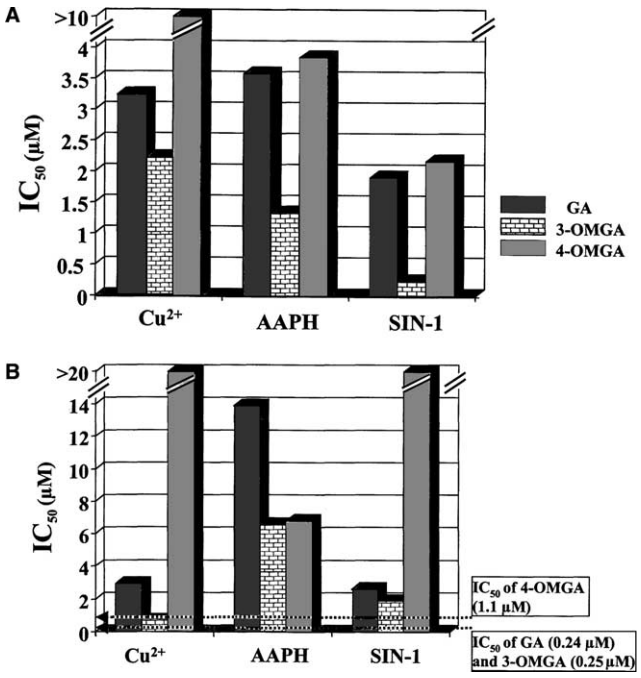

Fig. 1. Antioxidant capacities of the three forms of GA expressed as the concentration needed for a decreased LDL oxidizability by 50% (IC_{50}). For expression of the antioxidant capacities with the Cu^{2+}- and AAPH-oxidation systems, the lag time (T_{lag}) of LDL oxidation due to PP was first measured and compared to the T_{lag} without PP, leading to calculate a relative T_{lag} (rT_{lag}) [33], then the logarithm of $1/rT_{lag}$, which is plotted vs increasing PP concentrations, allowing to assess the IC_{50} value. For SIN-1-oxidation system, IC_{50} were obtained by a direct plotting of the molar ratio CD-production/apoB-concentration vs increasing PP concentrations. (A) Oxidation at conventional concentration (LDL $\leqslant 0.1$ μM). (B) Oxidation at near physiological concentration (1-μM LDL).

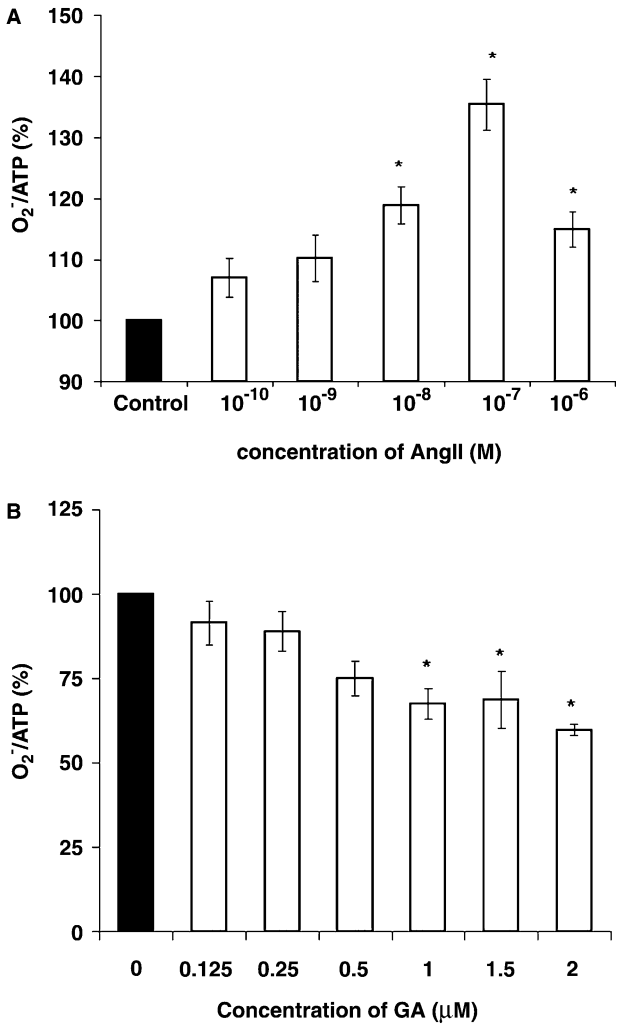

Fig. 2. Superoxide production in 72-h differentiated MDPs. (A) Effect of Ang II on the PMA-stimulated $O_2^{\cdot-}$-production. Cells were incubated with Ang II for 24 h, PMA (0.1 μM) was then added for a time period (90 min) during which the lucigenin-chemiluminescence was measured. Data were from seven separate experiments. (B) Effect of gallic acid on the $O_2^{\cdot-}$-production in the 0.1-μM Ang II-treated MDPs. Data were from four separate experiments. In A and B, the values for each experiment corresponded to the means of three determinations. $^*P < 0.05$ vs control.

highlight that the conditions of LDL concentration for assessing antioxidant capacity were of prime importance. In addition, it is worth comparing the IC_{50} values of the three forms obtained in the more physiological condition to their actual concentrations in plasma as reported by labels in Fig. 1B. We found that neither GA nor 4-OMGA had IC_{50} values compatible with an antioxidant effect in plasma regardless of the oxidation system. In contrast, the IC_{50} value of 3-OMGA was low enough (0.24 μM) to be protective towards Cu^{2+} oxidation at circulating concentration in plasma (0.25 μM).

In PMA-stimulated MDPs, an increase in $O_2^{\cdot-}$-production was observed with the differentiation time. The highest production was found for 72-h differentiation (not shown). We chose this differentiation time for all the experiments. After an additional 24-h incubation time with increasing Ang II concentrations, the PMA-stimulated MDPs showed (Fig. 2A) an $O_2^{\cdot-}$-production which culminated at 0.1 μM, corresponding to a 35% increase when compared to the no Ang II-treated MDPs. We next examined whether PP exhibit modulating effects on the $O_2^{\cdot-}$-production of Ang II-treated, PMA-stimulated MDPs. Fig. 2B clearly showed that growing concentrations of GA (in a concentration range of 0.012 μM to 2 μM) were able to increasingly inhibit $O_2^{\cdot-}$-production. The inhibition peak value (near 50%) reached a plateau for 1–2 μM GA. It is worth noticing that 0.25 μM GA had no significant effect. Interestingly, no inhibition was found with 3-OMGA and 4-OMGA at any concentration (not shown).

Fig. 3A shows that the expression peak of CD36 mRNA was obtained after a 72-h differentiation time, with an expression increasing by 67% as compared to non-differentiated cells. Therefore, the following experiments were carried out on 72-h differentiated MDPs. In one case, we added PMA in conditions which were similar to those used for superoxide production, i.e., at the concentration of 0.1 μM and with an incubation time of 90 min (Fig. 3B). This resulted in a CD36 mRNA expression even more pronounced. On the other hand, incubating Ang II for 24 h dose-dependently increased the CD36 mRNA expression (Fig. 4). In our hand, the highest expression was obtained for 0.1 μM Ang II. This action appeared to be similar to that of 0.1 μM PMA. As illustrated by Fig. 5, GA, 3-OMGA and 4-OMGA were able to

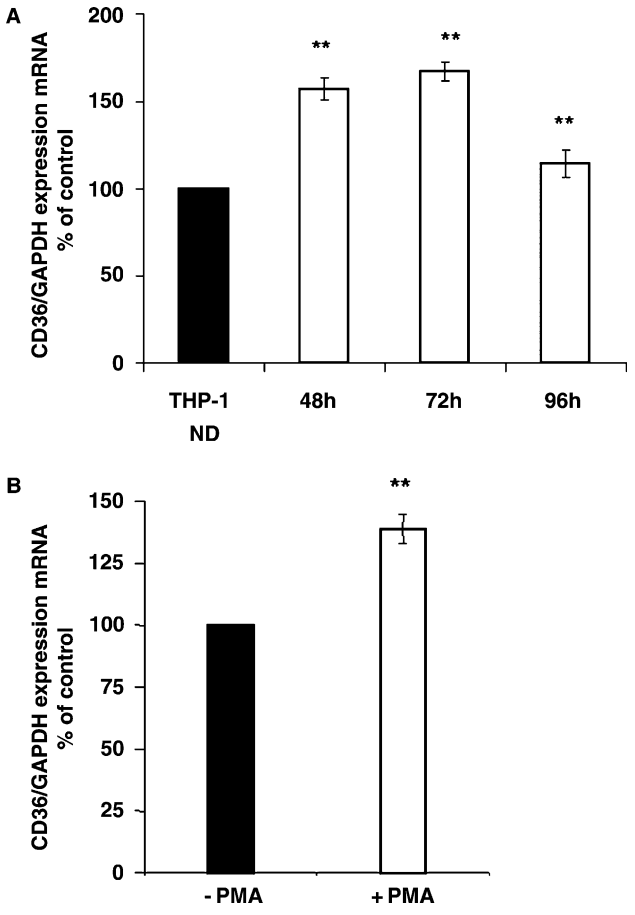

Fig. 3. Relationship between CD36 mRNA expression and differentiation time of THP-1. Cells were differentiated by retinoic acid, IFNγ and vitamin D3 for 48, 72 or 96 h. After total RNA isolation, the expression of CD36 mRNA was analyzed by Northern blot at each time point. Its expression was normalized by GAPDH mRNA expression. Data (means ± S.D.) were given for three separate experiments. (A) CD36 mRNA expression as a function of time. (B) In 72-h differentiated MDPs, an additional 90-min incubation with 0.1-μM PMA led to increase the CD36 mRNA expression. $^{**}P < 0.01$ vs non-differentiated THP-1.

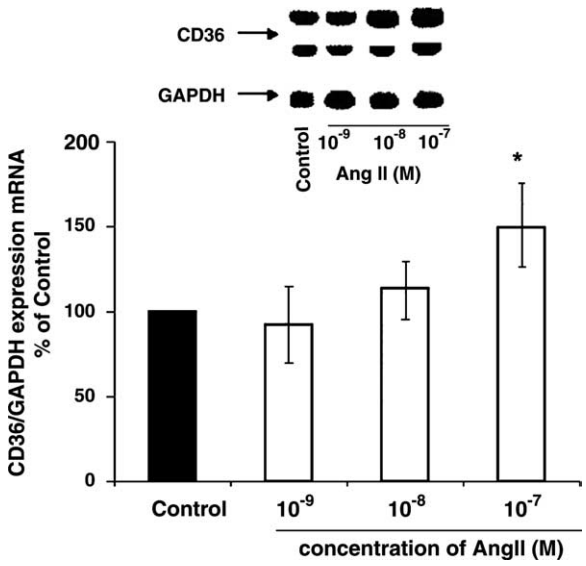

Fig. 4. Ang II enhances CD36 mRNA expression in MDPs. After a 72-h differentiation time, cells were incubated with increasing doses of Ang II for 24 h. Data (means ± S.D.) were given for three separate experiments. $^{*}P < 0.05$ vs control.

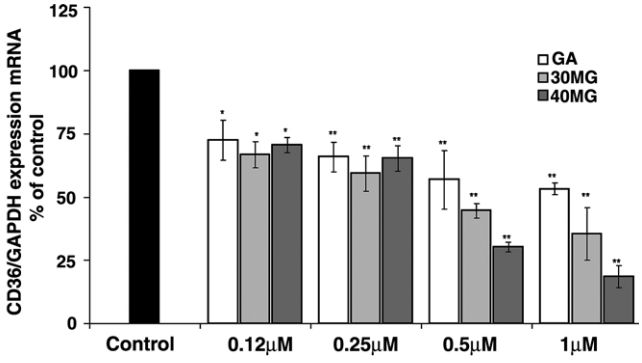

Fig. 5. Effects of the three forms of gallic acid on the CD36 mRNA-expression in MDPs. Cells were treated with Ang II (0.1 μM) in the presence or absence of GA, 3-OMGA or 4-OMGA. Total RNA was isolated, Northern blot analysis for CD36 mRNA was quantified by means of a PhosphorImager and normalized by GAPDH mRNA. Data (means ± S.D.) were given for three separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ vs control (in the absence of PP, but in the presence of Ang II and solvent vehicle).

dose-dependently inhibit the expression of CD36 mRNA. A 47%, 65% and 82% inhibition of CD36 mRNA expression was obtained for 1 μM GA, 3-OMGA, and 4-OMGA, respectively. In the same condition of PP concentration, the inhibition of AT1R mRNA expression was of 34%, 38% and 84%, respectively (Fig. 6).

4. Discussion

We have recently showed that the circulating concentrations of GA and their *O*-methyl derivatives in blood do not exceed 0.25 μM for GA and 3-OMGA, and 1.1 μM for the 4-OMGA (Fig. 1B) after drinking a moderate volume (300 ml) of red wine [30]. It will be of interest to compare these concentration values to those revealing a biological action.

We show here that the biological properties of GA metabolites presently studied were different and they were also different from those of GA. This was true for the direct antioxidant properties, for the regulation of superoxide production and for the mRNA expression involved in foam cell formation (CD36 mRNA, in particular) in MDPs.

Considering the circulating concentrations of GA and GA derivatives, it is shown that physiological concentrations were not able to significantly prevent LDL oxidation in the two in vitro oxidation systems (AAPH and SIN-1) considered to be relevant from a pathophysiological viewpoint (see [33] for discussion of this point). This does not mean, however, that these polyphenolic compounds are totally devoid of antioxidant properties. Likewise, physiological concentrations of GA were not able to significantly impair the superoxide cell production (Fig. 2A), whereas 3-OMGA and 4-OMGA did not exhibit any regulatory action at any concentration tested.

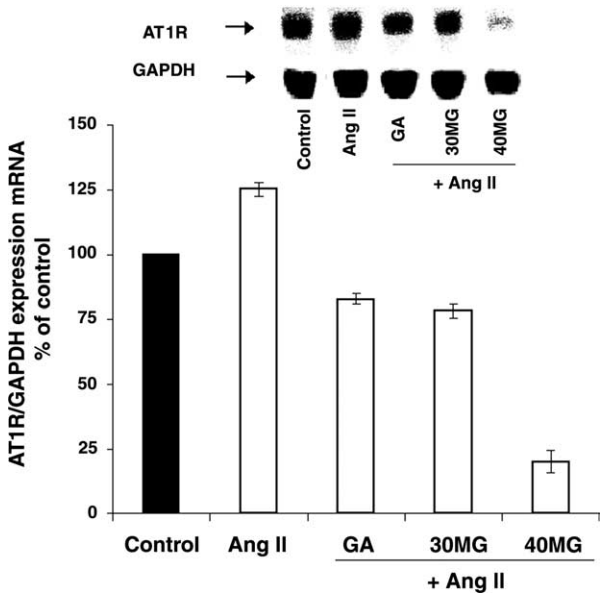

Fig. 6. Effects of the three forms of GA on the AT1R mRNA expression in MDPs. Cells were treated with Ang II (0.1 μM) in the presence or absence of GA, 3-OMGA or 4-OMGA at a concentration of 1 μM. For other information, see Fig. 5. $^{*}P < 0.05$; $^{**}P < 0.01$ vs Ang II only.

However, it would be of interest to test whether these PP are ineffective antioxidants at physiological concentrations in the presence of cell-released superoxide, which is the most suitable approach to assess both the direct radical-scavenging and the released-radical impairing due to PP [36].

In contrast, physiological concentrations of 4-OMGA – and to a lesser extent those of GA and 3-OMGA – were able to considerably depress the CD36 and AT1R mRNA expression in MDPs. In fact, we show for the first time that Ang II enhanced the expression of these two mRNAs in THP-1-derived macrophages and that 4-OMGA was not only able to counteract this action of Ang II, but also to decrease the mRNA expression beyond the one observed without Ang II, suggesting that the mechanism of action of this PP is not purely restricted to its role as modulator of AT1R mRNA expression. The 4-OMGA property presently found by us is of particular interest, especially as (i) the renin–angiotensin system is upregulated during THP-1 differentiation [14] and (ii) Ang II is known to exhibit a promigratory action on THP-1 monocytes [37], which contributes to its proatherogenicity. On the other hand, these results also showed that Ang II may not only increase CD36 expression by promoting oxLDL formation [8] through the increased superoxide production [11], but also directly interfere with the regulatory pathways involved in macrophage CD36 expression.

Altogether, these findings strongly suggest that the biological properties presently found are not explained by the direct antioxidant properties of GA and its *O*-methyl derivatives. Indeed, the less active form as antioxidant, 4-OMGA, was the most active towards the decrease in the CD36 and AT1R mRNA expressions.

Generally speaking, and regardless of the concentration conditions, 3-OMGA was the most antioxidant form of GA. The antioxidant capacity of GA was, however, close to that of 3-OMGA with both the Cu^{2+} and SIN-1 oxidation systems. This is in accordance with their highly antioxidant orthodiphenol structure which, in contrast, is disrupted in 4-OMGA. However, the very low antioxidant capacity of GA with the AAPH oxidation system remains to be explained. In contrast, the discrepant results of Fig. 1A and B – obtained in conventional (non-physiological) and more physiological conditions of concentration, respectively – underline the need for a critical examination of the conditions generally used for assessing antioxidant protection of LDL.

In VSMC, Ang II signaling has been shown to depend on the PKC/MAPK-mediated expression of AT1R via an AP-1 element (probably c-Fos) regulation [38,39]. The present results obtained in a model of macrophage could be explained partly by the GA potency to depress AP-1 activity [40]. There is also a possibility that PKC activity was impaired by GA as suggested by the phenolic-acid impairing of PKC-dependent superoxide production already found by our group [36]. However, the Ang II action is also mediated by the Rho-protein/Rho-kinase/NFκB pathway [41] and by a tyrosine kinase pathway [42], on which PP effects remain to be thoroughly explored. On the other hand, considering the Ang II capacity at mediating the oxLDL uptake, the present results show that this bioactive peptide is able to enhance expression of LOX-1 [43,44] and CD36 as well – as presently suggested by the increased expression of its mRNA – both known as oxLDL receptors.

It is worth noting that PMA-stimulated superoxide production was potentiated by pre-incubating MDPs with Ang II. The same is true for the increased CD36 mRNA expression due to incubating MDPs with PMA once cells were differentiated with the differentiating mixture (Fig. 3B). These results provide evidence that PMA kept on its stimulating and differentiating properties in the present conditions of differentiation (using the RDI mixture). It is indeed well known that prolonged cell-incubation with PMA (generally for 12 h) usually employed for cell differentiation leads to PMA-insensitive THP-1 cells [45].

Interestingly, 4-OMGA was found to be the most potent form to depress AT1R and CD36 mRNA expression. Blocking the AT1R pathway by a pharmacological approach through the angiotensin converting enzyme inhibition as carried out in ramipril-receiving mice has been shown to result in impairing CD36 mRNA expression in macrophages [46]. Using another approach, we found here a similar result.

In conclusion, among the three forms of GA presently studied, the 4-*O*-methyl derivative of GA appears to be the most potentially effective one in preventing Ang II-triggered foam cell formation. Its mechanism of action may imply depressing effects on both AT1R and CD36 mRNA expressions. Further studies on the corresponding-protein expression and on animal model are needed to confirm the anti-atherogenic effect of GA metabolites and to provide clear mechanistic evidence for an atheroma-formation preventing role of a PP-rich diet.

Acknowledgements: The program CAPES/COFECUB (Coordenaçao de Aperfeiçoamento de Estudo Superior and Comité Français d'Evaluation de la Coopération Universitaire avec le Brésil) No. 262/98-II has played a significant part in this work by attributing a fellowship to Vilma Bizerra. We are grateful to both organizations for their contribution.

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