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cis- and *trans*-Resveratrol Are Glucuronidated in Rat Brain, Olfactory Mucosa and Cultured Astrocytes

Nicole Sabolovic Tony Heurtaux Anne-Claude Humbert Stéphanie Krisa
Jacques Magdalou

UMR 7561 CNRS-Université Henri Poincaré Nancy 1, Faculté de Médecine, Vandœuvre-lès-Nancy,
et Université Victor Segalen, Bordeaux, France

Key Words
Resveratrol · UDP-glucuronosyltransferases · Glucuronidation · Olfactory mucosa · Astrocytes

Abstract
Background/Aims: Glucuronidation of *cis*- and *trans*-resveratrol (3,5,4′-trihydroxy-trans-stilbene), which is a naturally occurring phytoalexin known to exert a number of beneficial health effects, was investigated in rat brain, cultured astrocytes and olfactory mucosa. ***Methods:*** The isomers were incubated with tissue homogenates, microsomes, or rat liver recombinant UDP-glucuronosyltransferases in the presence of UDP-glucuronic acid. The glucuronides were separated by HPLC and quantitated. Astrocytes were exposed to lipopolysaccharide to promote inflammatory conditions. ***Results:*** All tissues were able to form resveratrol glucuronides although at a lower extent, when compared to the liver. The reaction was stereo- and regioselective. In brain tissue, trans-resveratrol 3-*O*-glucuronide was mainly formed, whereas the *cis*-isomer was glucuronidated at a lower rate on that position. No 4′-*O*-glucuronide was detected in brain. In olfactory mucosa homogenates, the cis 3-*O*-glucuronide was mainly formed, whereas the *trans*-isomer was glucuronidated only on the 3-position. In astrocytes, 3-*O*-glucuronides of the *cis*- and *trans*-resveratrol were only detected. The rat recombinant UGT1A6 and UGT2B1 isoforms were able to glucuronidate *cis*- and *trans*-resveratrol. Finally, in inflammatory conditions, *trans*-resveratrol glucuronidation was enhanced in astrocytes. ***Conclusion:*** Brain tissues are effective in the glucuronidation of resveratrol isomers. This metabolism pathway is likely to modulate the concentration of these biologically active substances.

Introduction

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a naturally occurring phytoalexin found in a variety of plant sources, such as peanuts, grapes and particularly found in high concentrations in red wine [1–3].

There is considerable research interest to determine the therapeutic potential of resveratrol, as it has been suggested to exert various beneficial properties in terms of cardioprotection, anticancer and neuroprotective effects. Thus, resveratrol has been shown to reduce the incidence of coronary heart disease [4]. Both in acute experiments and in chronic models, reveratrol was shown to attenuate myocardial ischemic reperfusion injury and atheroscle-

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Dr. Nicole Sabolovic
UMR 7561 CNRS-Université Henri Poincaré Nancy I, Faculté de Médecine
BP 184, FR–54505 Vandoeuvre-lès-Nancy, Cedex (France)
Tel. +33 383 68 39 60, Fax +33 383 68 39 59
E-Mail nicole.sabolovic@medecine.uhp-nancy.fr

rosis, and to reduce ventricular arrhythmias. The resveratrol cardioprotection property is achieved through a preconditioning effect rather than direct protection [5]. Resveratrol was also reported to have anticarcinogenic activities, preventing tumor initiation, promotion, and progression [6–10].

Interestingly, there is a growing number of studies indicating that natural polyphenol extracts may reduce the incidence of age-related neurological disorders including dementia [11, 12]. A protective effect of resveratrol was shown on beta-amyloid (Aβ)-induced oxidative PC12 cell death [13]. Resveratrol, which enters rapidly the central nervous system, has been found to protect brain against damages [14, 15] and to protect hippocampal cells against Aβ-induced toxicity through its ability to rapidly activate protein kinase C [16]. More recently, it has been suggested that resveratrol and flavonoids exert neuroprotective effects in various models of toxicity [17, 18]. The presence of specific resveratrol plasma membrane binding sites was detected in rat brain [15].

It has been reported that resveratrol, like other polyphenols, is extensively metabolized via the glucuronidation pathway in humans [19]. The reaction is catalyzed by the UDP-glucuronosyltransferases (UGT), a multigenic family of enzymes that transfer a glucuronic acid moiety from the donor substrate, UDP-glucuronic acid, to the phenolic groups of the substrate, leading to the formation of *O*-glucuronides that are readily excreted in urine [20]. We recently reported that human liver microsomal UGTs could actively glucuronidate both the *cis*- and *trans*-isomers of resveratrol [21]. The reaction was regioselective, leading to the formation of 3-*O*- and 4′-*O*-glucuronides but no 5-*O*-glucuronides. We found that the UGT1A1 glucuronidated the *trans*-resveratrol and the UGT1A6 glucuronidated the *cis*-resveratrol, whereas UGT1A9 glucuronidated both isomers. By contrast, UGT 2B family isoforms, especially UGT2B7, glucuronidate these substances at a very low extent [21].

Since we have identified several UGT isoforms in brain tissues and in olfactory mucosa [22, 23], it was tempting to determine the metabolism of resveratrol and of the *cis*-isomer also naturally present in these tissues. Thus, glucuronidation activities may strongly affect the concentration of these substances. Particularly, resveratrol glucuronidation was measured in the most abundant cells of the central nervous system, the astrocytes. Moreover, in the brain, glucuronidation is completed mainly by astrocytes which exhibit the highest UGT1A6 activity and expression among brain cells [22].

Materials and Methods

Chemicals

UDP-glucuronic acid was purchased from Boehringer Mannheim (Mannheim, Germany). *trans*-Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) was purchased from Sigma-Aldrich (St Quentin Fallavier, France). The *cis*-isomer was obtained by β-glucuronidase hydrolysis of *cis*-piceid, with a purity of 95%. *cis*-Piceid was isolated from *Vitis vinifera* cell suspension cultures by a combination of chromatographic techniques, and characterized by spectrometric methods, as described previously [24]. D-Saccharic acid 1,4-lactone (saccharonolactone), β-glucuronidase (bovine liver, 1×10^6 units/g) and lipopolysaccharide (LPS, from *Escherichia coli* 0111:B4) were from Sigma-Aldrich. Trifluoroacetic acid and dimethylsulfoxide were obtained from Merck (Darmstadt, Germany), and acetonitrile was from Carlo Erba (Val de Reuil, France). All other reagents were analytical grade and obtained from Sigma. Cell culture media were provided by Gibco (Cergy-Pontoise, France).

Animals

This study was performed with Wistar rats (Iffa Credo, Saint-Germain-sur l'Arbresle, France). The animals were maintained under standard laboratory conditions on 12/12 h light/dark cycles, with unrestricted access to standard chow and tap water. The study was approved by the local animal ethics committee, and all procedures adhered to the Principles of Laboratory Animal Care.

V79 Cells Expressing Rat UGT Isoforms

The rat recombinant UGT1A6 and 2B1 were stably expressed in the Chinese hamster lung fibroblasts V79 cell line as previously described [25, 26]. Expression level was monitored by Western blot, and activity was measured using 4-methylumbelliferone and ketoprofen as marker substrates of UGT1A6 and UGT2B1, respectively [27, 28].

Brain, Olfactory Mucosa and Liver

The rats were sacrificed by decapitation and the brains were rapidly removed, whereas olfactory mucosa was carefully scraped under a binocular microscope to remove any cartilage debris, in sterile cerebrospinal medium (124 mmol/l NaCl, 3.3 mmol/l KCl, 1.24 mmol/l KH_2PO_4, 1.3 mmol/l $MgSO_4$, 2.5 mmol/l $CaCl_2$, 26 mmol/l $NaHCO_3$, 10 mmol/l glucose).

Liver samples were also obtained from 3-month-old rats. They were immediately frozen by immersion into liquid nitrogen and stored at –80°C until homogenates (for olfactory mucosa) and microsomes (for brain and liver) preparations were made up.

Primary Cultures of Astrocytes

Astrocytes were prepared from newborn Wistar rat brains, as described previously [29]. Precisely, rats were sacrificed by decapitation and the brains were rapidly removed and placed in the Dulbecco's modified Eagle's medium (DMEM) containing 2 mmol/l L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin and 10% (v/v) heat-inactivated fetal calf serum. Residual meningeal tissues were removed from brains using fine scissors and tweezers.

The cells were dissociated in the culture medium by passing through a hypodermic syringe (21G, 0.8 × 25 mm) and centri-

fuged at 200 *g* for 10 min. The resulting pellet was resuspended in culture medium and plated on 75 cm^2 flasks at a density of 2 brains per flask. The cells were grown in a humidified atmosphere of 5% CO_2 at 37°C, and the culture medium was renewed twice a week. All experiments were carried out in Petri dishes, on cell subcultures at passage 2. Cell culture purity was characterized by the immunocytochemical detection of a specific astrocyte marker, the glial fibrillary acidic protein, as previously described [30].

Astrocyte Treatments with LPS

To promote inflammatory conditions, primary confluent cell cultures were treated with different concentrations of LPS (0.1, 1 and 10 μg/ml) diluted in PBS and added to the culture medium for 12, 24 and 48 h, at 37°C and 5% CO_2 [31]. After treatments, viability was estimated. The mitochondrial function was assessed as an index of cellular viability using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method, as previously described [30].

Preparation of Homogenates and Microsomes from Tissues and Cultured Cells

Homogenates of olfactory mucosa were used because small quantity of mucosa was available from a rat, making difficult to isolate microsomes. Samples were thawed and homogenized in a 50-mmol/l potassium phosphate buffer (pH 7.4), 1 mmol/l EDTA, 0.1 mmol/l dithiothreitol using a manual glass Dounce type homogeneizer. Brain microsomes were prepared according to the method of Dragacci et al. [32].

Astrocytes microsomes were prepared from cells collected on Petri dishes. After washing twice with phosphate-buffered saline (PBS), the pellets were resuspended in 50 mmol/l potassium phosphate buffer (pH 7.4) complemented with 1 mmol/l EDTA and 0.1 mmol/l dithiothreitol. They were sonicated in ice three times for 10 s at 60 W output power. The homogenates were centrifuged at 10,000 *g* for 20 min and the supernatants were further centrifuged at 100,000 *g* for 60 min. The microsomal pellets were suspended using a manual glass-glass Dounce-type homogenizer in a 100 mmol/l potassium phosphate buffer (pH 7.4), 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 20% (v/v) glycerol, and stored at –80°C until use.

Protein Content Measurements

Protein content of the homogenates or microsomal fractions was determined by the technique of Bradford [33] using a kit from BioRad (Hercules, Calif., USA) with bovine serum albumin (fraction V, Sigma-Aldrich) as standard.

Enzymatic Assay for Resveratrol Glucuronidation

Before activity assays, the brain and astrocytes microsomes were activated by addition of 0.2 mg Triton X-100/mg protein for 15 min on ice [22] to obtain maximal enzyme activation.

Then, activated microsomes and homogenates (100 μg) were incubated with 1 mmol/l *cis*-or *trans*-resveratrol (dissolved in dimethylsulfoxide), in 100 mmol/l Tris-HCl buffer (pH 7.4), 10 mmol/l $MgCl_2$, 5 mmol/l D-saccharic acid 1,4-lactone and 3 mmol/l UDP-glucuronic acid.

The assays were done in triplicate and control assays without UDP-glucuronic acid were run simultaneously. After incubation at 37°C for 60 min, reactions were stopped by addition of 6 N HCl (10 μl). The precipitated proteins were removed by centrifugation at 4°C for 5 min at 8,000 *g*, and the supernatants were directly injected into the HPLC apparatus.

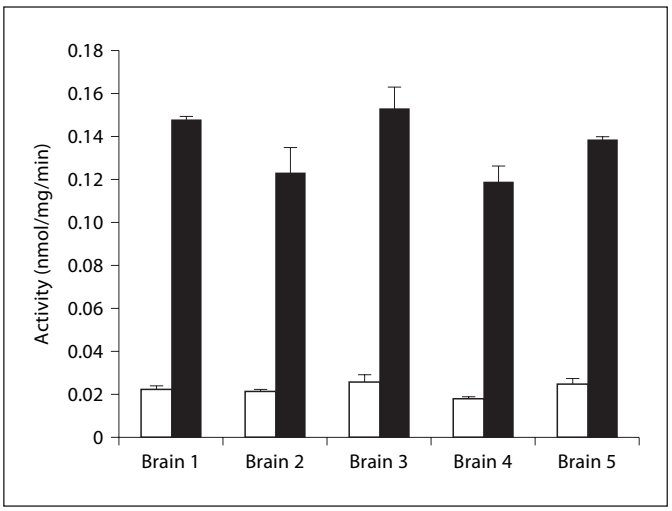

Fig. 1. Glucuronidation of the two resveratrol isomers in the rat brain. *cis*- and *trans*-Resveratrol (1 mmol/l) were incubated with microsomes (100 μg) prepared from rat brains. The formation of 3-*O*-*cis* and *trans*-glucuronides was plotted whereas the 4′-*O*-glucuronides were not detectable (limit of detection, 0.01 nmol glucuronide). The glucuronides were separated by HPLC as described in 'Materials and Methods'. The data presented are means ± SD of 3 separate determinations. □ = *cis*-3-*O*-glucuronide; ■ = *trans*-3-*O*-glucuronide.

The glucuronides were separated from the parent compounds on a Radial Pack C-18 reversed-phase column (10 × 111 mm) enclosed in a Waters RCM 100 radial compression module (Guyancourt, France), as previously described [21]. Briefly, elution was carried out with 20% (v/v) acetonitrile in water, containing 0.05% (v/v) trifluoroacetic acid (apparent pH 2.2). Peaks were detected respectively at 286 and 306 nm for *cis*- and *trans*-resveratrol, and quantitated by comparison to standard curves created using the parent compounds. The structure of the resveratrol glucuronides formed by human liver microsomes and recombinant UGTs was characterized by electron spray mass spectrometry and proton NMR as previously reported [21].

Results

Glucuronidation of Resveratrol in Rat Brain

Figure 1 shows the glucuronidation of resveratrol in rat brains. *cis*- and *trans*-Resveratrol were both conjugated in 3-*O*-glucuronides. However, a much higher rate for *trans*-conjugation was observed (×6), when compared to that of the *cis*-isomer. The formation of 4′-*O*-glucuronides was not detected.

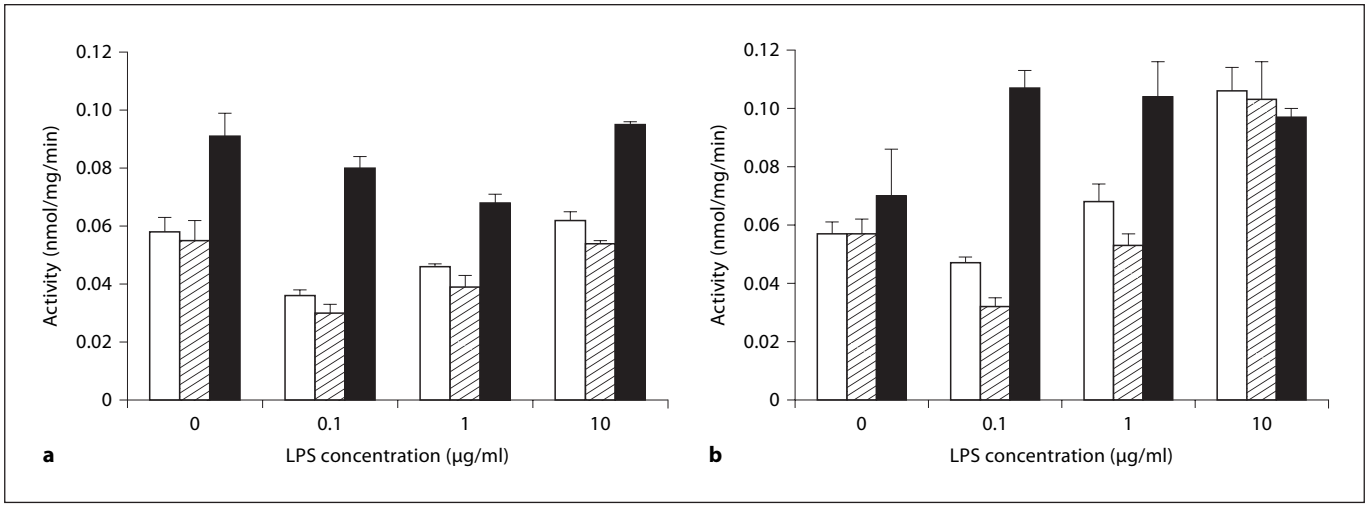

Fig. 2. Glucuronidation of *cis*- and *trans*-resveratrol by rat astrocytes with or without LPS exposure. Glucuronidation activity was measured in primary astrocytes cultures treated or not with LPS (0.1, 1 and 10 μg/ml) for 12, 24 and 48 h. The glucuronidation was evaluated by incubating microsomes (100 μg) with *cis*- and *trans*-resveratrol (1 mmol/l). Each value represents the means ± SD of 3 measurements. *cis*-3-*O*-glucuronides (**a**) and *trans*-3-*O*-glucuronides (**b**) after 12 h (□), 24 h (▨), and 48 h LPS exposure (■).

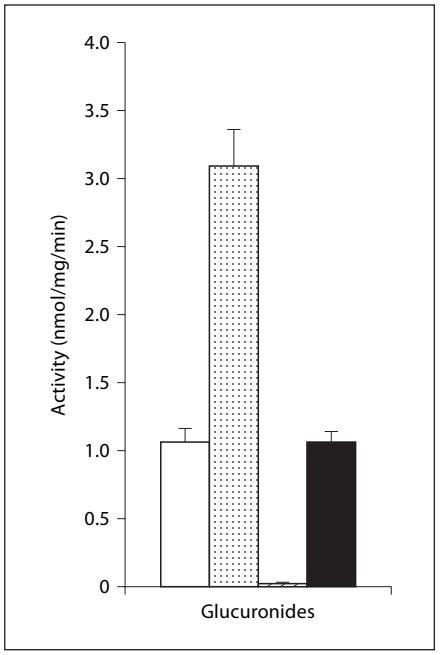

Fig. 3. Glucuronidation of resveratrol by tissue homogenates of rat olfactory mucosa. The amount of *cis*- and *trans*-glucuronides was measured by HPLC as described in 'Materials and Methods'. Each value represents the means ± SD of 3 determinations. □ = *cis*-4′-*O*-glucuronide; ▩ = *cis*-3-*O*-glucuronide; ▨ = *trans*-4′-*O*-glucuronide; ■ = *trans*-3-*O*-glucuronide.

Glucuronidation of cis- *and* trans-*Resveratrol by Rat Astrocytes under Inflammatory Conditions*

We have investigated the glucuronidation rate of resveratrol by rat astrocyte primary cultures. Astrocytes were able to glucuronidate *cis*- and *trans*-resveratrol (fig. 2) and led to the formation of 3-*O*-*cis*- and 3-*O*-*trans*-glucuronides at similar levels, while the 4′-*O*-glucuronides were not detected. To promote inflammatory conditions, cells were submitted for 12, 24 and 48 h to LPS treatments. Glucuronidation of *cis*-resveratrol, was not affected with 10 μg/ml LPS, while we observed it decreased with 0.1 and 1 μg/ml, whatever the period of treatment (fig. 2a).

Glucuronidation of *trans*-resveratrol decreased with 0.1 μg LPS exposition during 12 and 24 h, but increased at 48 h. With 1 μg/ml LPS, glucuronidation was highest at 48 h, and with 10 μg/ml LPS, it was increased whatever the period of treatment (fig. 2b).

Glucuronidation of Resveratrol in the Rat Olfactory Mucosa

A very high conjugation activity was observed in the rat olfactory mucosa, when compared to the brain, with formation of 4′-*O*- and 3-*O*-glucuronides of both isomers (fig. 3). The production of the 3-*O*-glucuronide was higher than that of the 4′-*O*-glucuronide, (×3 for the *cis*-, and ×40 for the *trans*-glucuronide). In contrast to the brain,

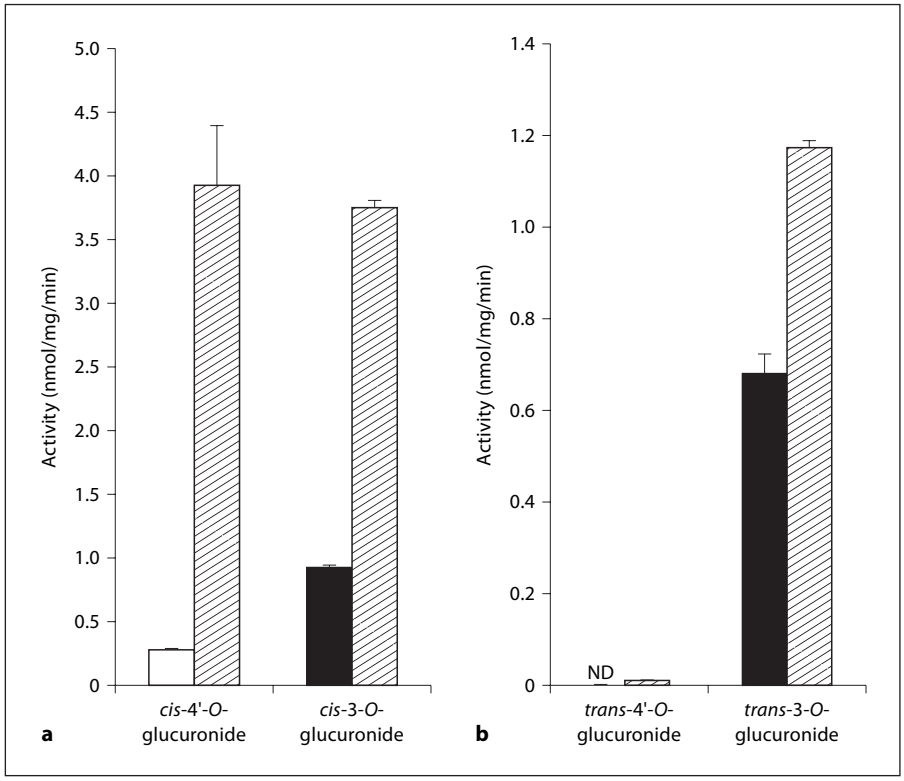

Fig. 4. Glucuronidation of resveratrol by rat liver microsomes. *cis*- and *trans*-resveratrol (1 mmol/l) were incubated with microsomes (100 μg) prepared from liver of rats treated by phenobarbital (PB) (▨) or not (□, ■). **a** Glucuronidation of *cis*-resveratrol. **b** Glucuronidation of *trans*-resveratrol. Each value represents the means ± SD of 3 determinations. ND = Not detectable.

the *cis*-resveratrol glucuronidation rate was very efficient, with a large production of *cis*-3-*O*-glucuronide in this tissue.

Glucuronidation of cis- *and* trans-*Resveratrol by Rat Liver Microsomes and Recombinant UGTs*

For comparison purpose, we determined the glucuronidation of *cis*- and *trans*-resveratrol by rat liver microsomes (fig. 4). Both isomers were effectively glucuronidated. However, if 4′-*O*- and 3-*O*-glucuronides were formed from the *cis*-isomer (fig. 4a), only 3-*O*-glucuronide of *trans*-resveratrol (fig. 4b) could be measured. Interestingly, treatment of rat with phenobarbital strongly induced the glucuronidation of *cis*- and *trans*-resveratrol. The formation of the 4′-*O*- and 3-*O*-glucuronides of *cis*-resveratrol was enhanced respectively by 10- and 4-fold, whereas that of 3-*O*-glucuronide of trans-resveratrol was only 2-fold increased.

We have also examined the glucuronidation of resveratrol by the rat liver recombinant UGT1A6 and UGT2B1 that have been stably expressed in V79 cells. The *cis*-resveratrol was conjugated by the two isoforms (fig. 5) leading essentially to the formation of *cis*-3-*O*-glucuronide by the UGT1A6 and of *cis*-4′-*O*-glucuronide by the UGT2B1. The production of *trans*-glucuronides was lower and the *trans*-4′-*O*-glucuronide was not detectable in the V79 cell lines expressing UGT1A6 isoform (fig. 5).

Discussion

The antioxidant, polyphenol, resveratrol detected in various natural sources including trees, flowering plants, peanuts and in grapevines, has been reported to possess numerous beneficial effects on human health in terms of anti-inflammatory, anti-atherogenic and anti-estrogenic actions. It has also been suggested that resveratrol presents anticancer and cardio- and chemopreventive activities. In addition, a possible neuroprotective effect has been postulated. Resveratrol was shown to protect neurons [34]. This action was mainly attributed to its antioxidant properties able to maintain cell viability by enhancing the intracellular free-radical scavenger glutathione [35]. Resveratrol can cross the blood-brain barrier and exert protective effects against cerebral ischemic injury in gerbils [36]. Additionally, resveratrol was shown to increase glutamate uptake and glutamine synthetase activity in C6 glioma cells. This

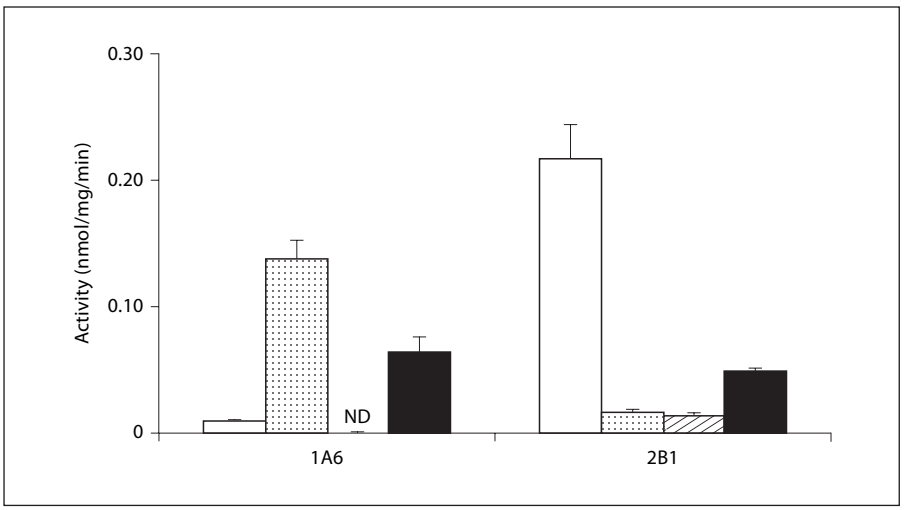

Fig. 5. Glucuronidation of *cis*- and *trans*-resveratrol by rat recombinant UGT isoforms. Microsomes isolated from V79 recombinant cell lines, stably expressing rat UGT1A6 or UGT2B1, were incubated with *cis*- and *trans*-resveratrol. Glucuronides were separated by HPLC as described in 'Materials and Methods'. Results are the means ± SD of 3 separate determinations. ND = Not detectable; limit of detection, 0.01 nmol glucuronide. □ = *cis*-4′-*O*-glucuronide; ▩ = *cis*-3-*O*-glucuronide; ▨ = *trans*-4′-*O*-glucuronide; ■ = *trans*-3-*O*-glucuronide.

property could contribute to the protective role of astrocytes in brain injury conditions [37]. Indeed, it has been hypothesized that a moderate consumption of tea or wine may reduce the risk of age-related neurodegenerative pathologies such as Alzheimer disease [18, 38, 39]. In the light of these observations, it is important to determine the fate of resveratrol in cerebral tissues, especially the contribution of the glucuronidation pathway in its biotransformation. Glucuronidation is the main phase II reaction leading to the detoxification and excretion of drugs. We and others presented evidence that resveratrol was extensively metabolized as glucuronides in major organs, including the liver and the gastrointestinal tract [19, 40]. The reaction was found to be regioselective, leading to the formation of various glucuronides (3- and 4′-*O*-glucuronides).

Although the glucuronidation reaction mainly occurs in liver, brain is also able to glucuronidate drugs and xenobiotics. We recently investigated the glucuronidation potency of this organ, along with olfactory bulb and olfactory mucosa. Thus, we have described the formation of drug glucuronides and the expression of the UGT1A6 isoform in these tissues [23, 41]. Moreover, it has been previously shown that, in the brain, glucuronidation was completed mainly by astrocytes which exhibit the highest UGT1A6 activity and expression among brain cells [22].

This work revealed that, indeed, microsomes prepared from brain and astrocytes, as well as homogenates of olfactory mucosa could glucuronidate *cis*- and *trans*-resveratrol. However, the glucuronidation rate was much more lower than that found in the corresponding liver. As we recently reported in human [21], the glucuronidation was regio- and stereoselective and leads to the formation of two glucuronides (3-*O*- and 4′-*O*-glucuronides). Attempts to identify the UGT isoforms involved in that process revealed that the 3-methylcholanthrene and the phenobarbital-inducible UGT isoforms, UGT1A6 and UGT2B1, respectively, which are the 2 main proteins identified so far in rat, were effective in the glucuronidation of the compounds. The expression of these isoforms which has been detected in astrocytes [unpubl. results] would account for this biotransformation.

On the other hand, it is known that inflammation is associated with various neurological injuries such as neurodegenerative disorders including Alzheimer and Parkinson diseases [42, 43]. We have also reported that cerebral metabolism could be altered under oxidative stress conditions [30] and neuroinflammatory states [31]. We investigated whether resveratrol glucuronidation in brain, could be affected under inflammatory conditions, and for this purpose, we submitted rat astrocytes primary cultures to LPS exposure. This endotoxin is known to produce reactive oxygen species which can promote protein degradation and activate transcription factors such as AP-1 and PPAR, which have been reported to modulate the expression of genes encoding UGT [44, 45]. However, more work is needed to better understand this process.

Interestingly, the olfactory mucosa was very active in glucuronidating *trans*- and *cis*-resveratrol, with specific activities similar to those measured in liver. Olfactory mucosa is known for expressing high amounts of UGT isoforms, especially UGT1A6, 2B1 and the tissue specific UGT2A1 [23, 41]. These enzymes are believed to partici-

pate to the olfactory process by odorant substance biotransformation, and act as a metabolic barrier against airborne hazardous chemicals.

In conclusion, this study shows that nervous tissues are able to glucuronidate *cis*- and *trans*-resveratrol in rat. Although the glucuronidation reaction occurs in lower rate, when compared to that measured in liver, the UGT expressed in nervous tissues can contribute efficiently to the biotransformation of these biological active substances.

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