

A functional link between heme oxygenase and cyclo-oxygenase activities in cortical rat astrocytes

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Abstract

Recent evidence shows that the activation of heme oxygenase (HO) within the CNS is associated with increased prostanoid production. In this study, we investigated whether changes in HO activity induced by pharmacological manipulation are associated with parallel variations in cyclo-oxygenase (COX) activity and prostaglandin production in an *in vitro* paradigm of CNS cells, i.e. primary cultures of rat cortical astrocytes. Pharmacological tools commonly used to induce changes in HO activity, namely the HO enhancers hemin and CoCl_2 as well as the HO inhibitor Sn-mesoporphyrin-9 (SnMP9), were tested in our model, and the variations in COX activity associated with the above treatments were monitored by measuring a COX end product, prostaglandin E_2 (PGE_2), released into the incubation medium. We found that the increase in HO activity induced by hemin and/or CoCl_2 was not consistently associated with increases in prostaglandin production, whereas HO inhibition by SnMP9 was normally followed by a decrease in PGE_2 release. The above effect was observed after both acute (30 min) and prolonged (24 hr) incubations, suggesting that baseline HO activity contributes to the maintenance of normal PG production in this model. Experiments with the stable HO end products biliverdin and bilirubin suggest that these products may play a role in mediating HO-induced COX activation. © 2001 Elsevier Science Inc. All rights reserved.

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1. Introduction

HO (EC 1.14.99.3) catalyzes the hydrolysis of heme groups released from the hemo-proteins, hemoglobin, myoglobin, cytochromes, etc, giving rise to equimolar amounts of iron, CO, and BV; the latter is further reduced to BR by biliverdin reductase [1]. Two HO isoforms have been characterized: a constitutive and an inducible isoform, HO-2 and HO-1 respectively. A third isoform has also been described, but has not yet been fully characterized [2]. Apart from the identity between their active centers, HO-1 and -2 strongly differ in their amino acid and nucleotide sequence, size, cell and tissue distribution, and regulation [1]. The CNS is endowed with very high levels of HO activity, in the order

of magnitude of those organs involved in hemoglobin degradation such as the spleen; under basal conditions, most OH activity is accounted for by HO-2 [3,4], but HO-1 can also be induced in glial cells by several stimuli including heat shock (indeed, HO-1 is also referred to as heat shock protein 32), heavy metals including cobalt, hemin itself, oxidative stress, and bacterial lipopolysaccharide. Such a phenomenon, i.e. the induction of HO-1 in cells of the glial lineage, is also commonly observed in *in vitro* paradigms [5–7].

At present, the role of HO in CNS physiology and pathology is still controversial, with some evidence showing protective effects against cell and tissue damage induced by various agents [7–9], and other reports suggesting that an increase in HO activity may have detrimental effects [5,10–13]. Responsible for such a possible dual role of HO are the end products of enzyme activity, i.e. iron, CO, BV, and BR. Indeed, CO is currently thought to be a gaseous neurotransmitter, with a modulatory role comparable to and overlapping with that of nitric oxide [14]; on the other hand, BR has long been known as a potential endogenous antioxidant

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Abbreviations: BR, bilirubin; BV, biliverdin; CHX, cycloheximide; CO, carbon monoxide; COX, cyclo-oxygenase; HO, heme oxygenase; INDO, indomethacin; LDH, lactate dehydrogenase; MEM, modified Eagle's medium; PGE_2 , prostaglandin E_2 ; and SnMP9, Sn-mesoporphyrin-9.

[15]. Moreover, increased free iron may cause damage through oxidative stress mechanisms [16,17].

We have previously shown that pharmacologically induced modulation of HO is associated with parallel fluctuations in COX activity in the rat hypothalamus *in vitro*, suggesting that the induction of prostanoid production is a biochemical event downstream of HO activation which might be responsible for certain biological activities exerted by this enzymatic pathway within the brain [18,19]. In the present study, a possible functional link between HO and COX activities was investigated in primary cultures of rat cortical astrocytes. A specific substrate, an inducer, and an inhibitor of HO were used as pharmacological tools to modulate enzyme activity, and the subsequent variations in COX activity were monitored by measuring a COX end product, PGE₂, synthesized by astrocytes and released into the incubation medium.

2. Materials and methods

2.1. Astrocyte cultures

Cultures of cortical type I astrocytes were obtained as described by McCarthy and de Vellis [20], with modifications. In brief, 1- to 2-day-old Wistar rats were decapitated. The brains were removed under aseptic conditions and placed in PBS (Sigma Chemical Co.) containing 100 IU/mL of penicillin and 100 µg/mL of streptomycin (Sigma). Under a stereomicroscope, the meninges were carefully removed and the cortex dissected. The tissue was cut into small fragments, digested with trypsin (EuroClone; 0.125% in PBS) for 25 min at 37°, and mechanically dissociated in Dulbecco's MEM with Glutamax-I (GIBCO BRL, Life Technologies) containing 100 IU/mL of penicillin, 100 µg/mL of streptomycin, and 10% fetal bovine serum (GIBCO) to obtain single cells. Viability was >90%. The cells were incubated at 37° in a humidified atmosphere containing 5% CO₂, the medium was changed after 24 hr and then twice a week. At confluence, the cultures were vigorously shaken to remove non-adherent cells (oligodendrocytes and microglia) and re-plated. This step was repeated 8–9 days thereafter to further purify the cultures; this protocol produced cultures with >95% glial fibrillary acidic protein-positive cells.

2.2. Experimental procedures

Astrocytes were grown to confluence in 24-well plates. The medium was changed 12 hr prior to the experiments with serum-free Dulbecco's MEM with Glutamax-I containing 0.2% BSA (Sigma), 100 IU/mL of penicillin, and 100 µg/mL of streptomycin, and again at the beginning of experiments with the same medium as above with graded concentrations of test substances and without antibiotics. The incubation times ranged between 30 min and 24 hr.

After the experiments, the medium was collected under sterile conditions and stored at –20° until PGE₂ assays were performed.

2.3. Analytical methods

2.3.1. Measurement of PGE₂

PGE₂ was measured by radioimmunoassay as previously described [21]. The detection limit of the assay was 2 pg/tube, and the EC₅₀ 28 pg/tube. The intra- and interassay variability was 5 and 10%, respectively.

2.3.2. LDH Assay

The assay was performed using a commercial kit (Abbott S.p.A) provided by Dr. P. Turno. Briefly, 33-µL aliquots of incubation media from astrocyte cultures were mixed with 1 mL of LDH reagent kept at room temperature. The rate of increase in absorbance at 340 nm caused by NADH formation was calculated as the difference between absorbance values recorded at 120 and 240 sec, with NADH formation being directly related to LDH activity. Data were expressed as IU of LDH/liter.

2.4. Drugs

Hemin HCl, BV, BR, CoCl₂, CHX, and INDO were purchased from Sigma; SnMP9 dichloride was obtained from Affiniti Research Products Ltd. CHX was dissolved in distilled water; hemin, BR, and SnMP9 were dissolved in 0.1 M NaOH; BV and INDO were dissolved in methanol and ethanol, respectively. All of the above substances were further diluted in Dulbecco's MEM with Glutamax-I. None of the substances tested interfered with the PGE₂ assay.

2.5. Statistical analysis

All results are means ± SEM. Data are expressed as pg of PGE₂/mL of incubation medium. Data were analyzed by one-way ANOVA followed by *post hoc* Bonferroni test for multiple comparisons among group means, using a Prism™ computer program (GraphPad). Student's *t*-test was adopted to check if the group means of LDH experiments were significantly different from zero. Differences were considered statistically significant if *P* < 0.05.

3. Results

Cortical rat astrocytes produced sizable amounts of PGE₂; the prostanoid was released into the incubation medium and did not appear to undergo any further metabolism or reuptake process, so that PGE₂ levels tended to increase in a time-dependent manner (Fig. 1).

In acute experiments (30-min incubations), the HO substrate hemin, up to 10 µM, did not modify PGE₂ production compared to controls. On the contrary, the HO inhibitor

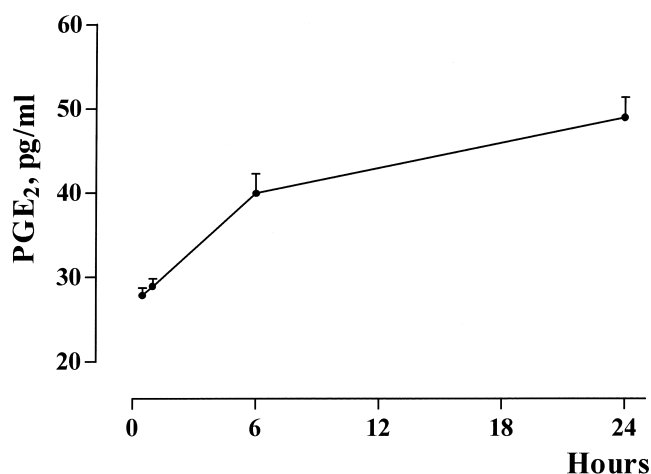


Fig. 1. The release of PGE₂ from rat cortical astrocytes increases linearly with time. Data are expressed as pg PGE₂/mL, the means \pm SEM of 6 replicates per group.

SnMP9, added to the incubation medium at 10 μ M, significantly reduced prostanoid production with respect to controls; this effect was partially reversed by hemin (Fig. 2).

In long-term (24 hr) experiments, hemin did not consistently modify PGE₂ production, although in some cases a significant increase was achieved (data not shown). In this series of experiments, a pure HO-1 inducer, CoCl₂ [22,23], was also tested. The latter, up to a 10- μ M concentration, failed to modify PGE₂ release with respect to controls (Fig. 3). Similar to acute experiments, SnMP9 was also able to significantly inhibit PGE₂ production after 24-hr incubation either alone or in the presence CoCl₂ (Fig. 3).

Thus, while the inhibition of HO by SnMP9 was clearly associated with a reduction in PG production, any attempt to increase such production by enhancing HO activity was unsuccessful, suggesting that our experimental paradigm is endowed with elevated COX activity already under basal conditions, perhaps as a result of COX-2 induction associated with culture technique [24,25]. To verify this hypothesis, we carried out experiments with the protein synthesis inhibitor CHX. The latter was used at 1 and 10 μ M in short

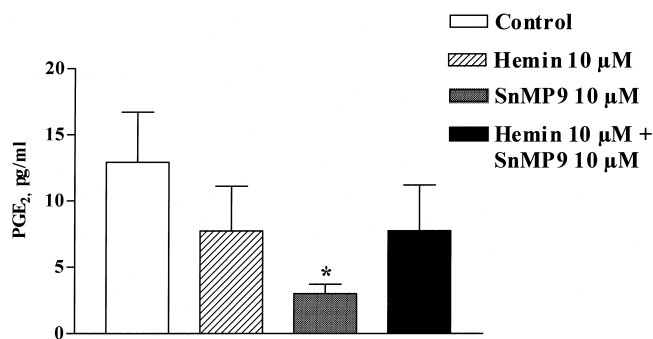


Fig. 2. The effects of hemin and SnMP9, alone or in combination, on PGE₂ release from astrocytes in 30-min experiments. Data are expressed as pg PGE₂/mL, the means \pm SEM of 10–12 replicates per group. *: $P < 0.05$ versus control.

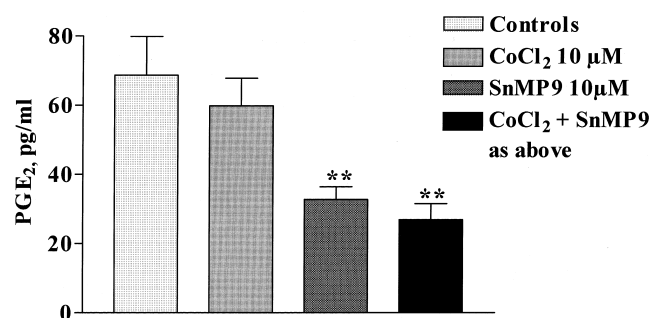


Fig. 3. The effects of CoCl₂ and SnMP9, alone or in combination, on PGE₂ release from astrocytes in 24-hr experiments. Data are expressed as pg PGE₂/mL, the means \pm SEM of 8 replicates per group. **: $P < 0.01$ versus control.

pulses (1 hr) before the 24-hr incubations. Fig. 4 shows that CHX inhibited PGE₂ release in a concentration-dependent manner, with statistical significance attained at 10 μ M.

Having observed that HO and COX activities were functionally linked in our experimental model, we attempted to identify the HO product(s), either CO or the stable end products BV and BR, which mediate HO-dependent prostanoid production. While technical difficulties prevented the direct use of CO, BV and BR could be tested in acute as well as 24-hr experiments. After both 30-min and 24-hr experiments, BR, but not BV, was able to increase PGE₂ production compared to controls; statistical significance was reached with the 10- μ M concentration (Fig. 5). The figure also shows that a non-selective COX inhibitor, INDO, completely abolished basal as well as BR-stimulated PGE₂ production.

In this study, a number of tools were tested at quite high concentrations, raising the question as to whether PGE₂ release might be influenced in a non-specific manner by toxic effects on astrocytes. We therefore conducted a cytotoxicity test, looking at LDH levels in the incubation medium. Table 1 shows that in no case (either after incubation in plain medium or in medium containing 10 μ M hemin, BV, or BR) was the LDH activity significantly different from zero.

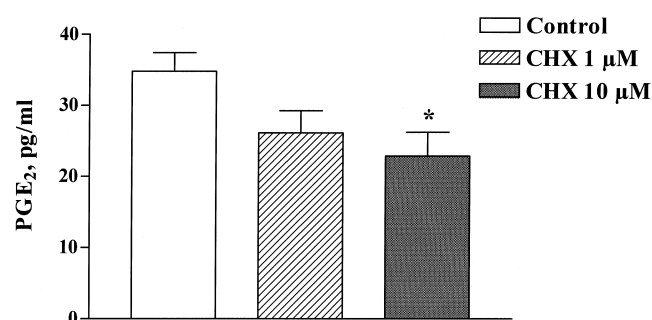


Fig. 4. The effect of 1-hr pretreatment with CHX on PGE₂ release from astrocytes in 24-hr experiments. Data are expressed as pg PGE₂/mL, the means \pm SEM of 21 replicates per group. *: $P < 0.05$ versus control.

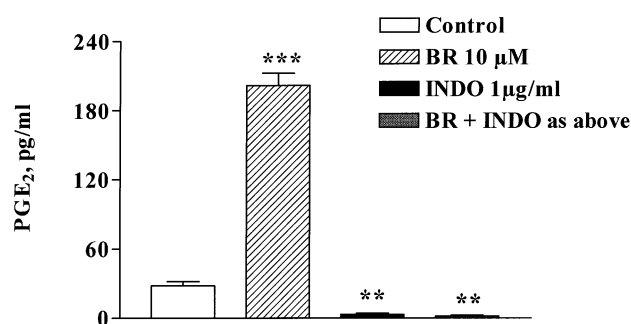


Fig. 5. The effect of 10 μ M BR, alone or in the presence of INDO, on PGE₂ release from astrocytes in 24-hr experiments. Data are expressed as pg PGE₂/mL, the means \pm SEM of 7–8 replicates per group. ** and ***: $P < 0.01$ and $P < 0.001$ versus control.

4. Discussion

Findings presented here indicate that a direct relationship between HO and COX activities exists in cultured rat cortical astrocytes, since the inhibition of HO activity was unambiguously associated with parallel decreases in prostaglandin production in these cells. On the contrary, we failed to induce significant and consistent increases in PGE₂ release by enhancing HO activity with hemin or CoCl₂. Taken collectively, these findings suggested that PG production in our experimental model is already elevated under basal conditions. Results of the experiments with the protein synthesis inhibitor CHX are consistent with this hypothesis, which is further confirmed by a number of reports showing that culture conditions per se are able to induce the gene expression and synthesis of COX-2 [23,24]. If inducible COX was expressed under long-term culture conditions, lower levels of activity (mostly accounted for by constitutive COX) would be expected to be present in acute explants of neural tissues. Indeed, in a short-term experimental paradigm, we found not only a reduction of PGE₂ release induced by HO inhibitors, but also an increased release caused by hemin after as early as 20 min of incubation [18].

Table 1
Levels of LDH activity in incubation media from astrocyte cultures

Treatment	LDH, U/L ($\mu \pm$ SEM, N)
Controls	0.63 \pm 0.41 (8)
Biliverdin, 10 μ M	0.63 \pm 0.41 (8)
Bilirubin, 10 μ M	0.94 \pm 0.46 (8)
Controls	0.84 \pm 0.53 (6)
Hemin, 10 μ M	0.84 \pm 0.53 (6)
Controls	1.26 \pm 0.56 (6)
Triton X-100 0.1%	45.30 \pm 0.65 (6)

The table also shows the effect of a treatment with Triton X-100, which has been used as a standard membrane disrupter on astrocyte cultures [30]. Results are expressed as international units/L, the means \pm SEM of (N) replicates per group.

Having shown that HO activity can influence PG production in our paradigm, we also attempted to clarify which HO end product is responsible for this effect, whether it be CO, the stable compounds BV and BR, or both. Incidentally, to our knowledge no study has been conducted thus far to investigate if astrocytes are endowed with BV reductase activity. In this study, our attention focused on stable end products, taking into account that BV and BR would accumulate in the system as the experiment went on, whereas CO would be cleared because of its volatile nature; this is true in particular for 24-hr incubation experiments. We used BR and BV at the same concentrations of hemin, i.e. up to 10 μ M, assuming that HO activity might degrade the total amount of hemin added to the system. While these concentrations are quite high, most probably higher than those reached *in vivo*, none of the above substances caused toxic damage to cell cultures, as assessed by the LDH assay. We found that BR, but not BV, was able to increase PGE₂ release after as early as 30 min of incubation and was still effective after 24 hr. In the absence of information concerning BV reductase activity in astrocytes, we cannot rule out the possibility that BV has no clear effect because it is reduced to BR in the system.

Although high concentrations of BR (such as those used in these *in vitro* experiments) are unlikely to be reached *in vivo* under normal conditions, this might occur during such disorders as neonatal jaundice associated with kernicterus [26]. In this case, BR is believed to be toxic for neurons, although more recent evidence has shown a protective role against hydrogen peroxide toxicity in hippocampal neurons *in vitro* [27]. However, these authors also pointed out that the neuroprotective effect is lost with increasing BR concentrations, and the highest doses used were toxic per se. Evidence presented here suggests that damage of neural tissue caused by elevated BR concentrations might be mediated by increased prostanoid levels.

In conclusion, an interesting interplay emerged in this study between the catabolism of heme moieties and the production of lipid mediators of the prostanoid series. It is quite difficult at this time to predict any pathophysiological relevance of these *in vitro* observations. Indeed, it is still a matter of debate whether HO has a protective or a damaging activity on CNS cells (see Introduction). Furthermore, increased PGE₂ production should not be taken as a mere index of inflammation, as there is convincing evidence showing a role for the prostanoid in the inhibition of inflammatory cells in the CNS [28,29].

Acknowledgments

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