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Hemoglobin Affects Lipid Peroxidation and Prostaglandin E_2 Formation in Rat Corticocerebral Tissues *In Vitro*

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ABSTRACT. Variations of lipid peroxidation and arachidonic acid (AA) metabolism products were found when experimental subarachnoid hemorrhage or ischemia and reperfusion were performed in an animal brain model. In a previous study, we showed that hemoglobin (Hb) produces prostaglandins when incubated in AA. To elucidate how Hb affects lipid peroxidation and AA metabolism in the CNS, we measured lipid hydroperoxides (LOOH), PGE_2 and thiobarbituric acid reactant substances (TBARS) in corticocerebral homogenates and slices of rats (normal rats) after incubation with different concentrations (10^{-9} to 10^{-5} M) of Hb. In addition, brain cortices of indomethacin-treated (40 mg/Kg) rats (IN-treated rat) were incubated in the presence of 10^{-5} M indomethacin (IN) to exclude the interference of prostaglandin enzyme synthetase. Hb was able to affect LOOH, PGE_2, and TBARS production in both normal and IN-treated rat brain cortex homogenates and slices. In all cases, we found an increase in prostaglandin when 10^{-8} M Hb was used, whereas no effect was noticed with 10^{-9} M. On the other hand, with higher Hb concentrations (10^{-6}–10^{-5} M), the LOOH and PGE_2 values did not reach statistical significance, and TBARS significantly increased. In all cases, when 10^{-4} M scavenger or metal-chelating compounds were added to an incubation mixture with 10^{-8} M Hb, PGE_2 formation was inhibited, whereas no variation occurred when 10^{-4} M IN was further added to IN-treated rat corticocerebral homogenate or slices. We hypothesize that in *in vivo* experimental neuropathologies, Hb must attain the 10^{-6} M concentration in the reaction cellular microenvironment to stimulate PGE_2 production, and that an evaluable part of this PGE_2 production may be directly ascribable to the iron-heme oxy-redoxy activity of Hb. BIOCHEM PHARMACOL 52;1: 97–103, 1996.

KEY WORDS. lipid peroxidation; prostaglandin; CNS; hemoglobin; free radicals; indomethacin

Experimental cerebral subarachnoid hemorrhage and ischemia followed by reperfusion can significantly modify biochemical parameters in brain tissue, such as lipid peroxidation, liberation of free radicals, changes in scavenger enzyme activity, and enhancement of AA† metabolism involving variation in PG concentrations [1–4].

Because injected and/or extravased blood is present in both neuropathologies, hemoglobin released by erythrocytes appears to be involved in the modification of biochemical parameters [5, 6]. In fact, hemoglobin elicits lipid peroxidation and release of PG when injected in subarachnoid space [7, 8].

In previous papers, we have shown that aerobic incubation of AA with peroxidase (E.C. 1.11.1.7) and other hemoproteins, in particular Hb, gives rise to the formation of a number of compounds with PG-like activities. Among these, PG E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) can be identified by thin layer chromatography and gas chromatography-mass spectrometry analysis.

PGE_2-like substance formation catalyzed by Hb is inhibited by deferoxamine and D-penicillamine, whereas IN is ineffective [9, 10].

Other researchers have found that, at low concentrations, Hb catalyzes a quasi-lipoxygenase reaction on linoleic acid, showing a remarkably high substrate specificity [11].

The present study was undertaken to elucidate how Hb affects lipid peroxidation and PGE_2 formation in the rat brain cortex. Therefore, we evaluated LOOH formation in normal rats as an index of the initial stages of lipid peroxidation and as a forerunning event of PG formation. PGE_2, as a metabolite of AA and TBAR production, as lipid peroxidation end products, were analyzed.

To assess the direct and effective ability of Hb to induce lipid peroxidation and PGE_2 formation, PG enzyme synthetase (PES) activity was inhibited by pretreating rats with IN.

Free radical and oxy-redoxy iron-heme activity were also

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† *Abbreviations:* CNS, central nervous system; Hb, hemoglobin; LOOH, lipidhydroperoxide; TBARS, thiobarbituric acid reactant substances; MDA, malondialdehyde; PG, prostaglandin; PES, prostaglandin enzyme synthetase; IN, indomethacin; AA, arachidonic acid.

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investigated by means of scavenger and/or iron-chelating agents such as DL-α-tocopherol [12], deferoxamine [13], D-penicillamine [14] and 21-aminosteroid U75400A [15], known to be effective both *in vivo* and *in vitro* in reducing lipid peroxidation in brain tissue.

MATERIALS AND METHODS

Materials

Rat hemoglobin (lyophilized powder prepared from washed, lysed, and dialyzed erythrocytes), IN, deferoxamine mesylate, DL-α-tocopherol (Sigma, St. Louis, MO, U.S.A.); bovine albumin (Merck, Darmstadt, Germany); C18 silica column (Varian, Harbor City, CA, U.S.A.); prostaglandin E_2 (^{125}I) RIA kit (NEN Chemicals, GmbH Dreiech, Germany); D-penicillamine (Eli Lilly, Indianapolis, IN, U.S.A.); 21-aminosteroid U74500A (Upjohn, Kalamazoo, MI, U.S.A.); and Wistar rats from Morini (Reggio Emilia, Italy). All the reagents were of the highest analytical grade commercially available.

Experimental Conditions

Male Wistar rats (250–300 g) were selected at random, divided into 2 groups and starved for 16–18 hr prior to treatment. Water was provided *ad lib*. One group did not receive any treatment (normal rats), whereas another group (IN-treated rats) was administered 40 mg/Kg of indomethacin by gavage 1 hr and ½ hr before sacrifice. All groups always consisted of 6 rats. All animals were killed by cervical dislocation and the cerebral cortices isolated from the removed brain [16].

Homogenate Incubation

Cerebral cortices of normal rats were homogenized (1% wt/v for PGE_2 and 5% wt/v for LOOH and TBARS evaluation) using a Polytron homogenizer (Kinematica CH) for 30 sec at 0°C in phosphate buffer (50 mM K_2HPO_4-KH_2PO_4 in 0.142 M NaCl) pH 7.4. When cerebral cortices of IN-treated rats were used, the homogenization was performed in the presence of 10^{-5} M indomethacin.

Aliquots (0.5 mL) of homogenate were incubated in polypropylene tubes at 37°C in water bath for 1 hr under agitation, together with 0.1 mL of rat Hb solution (10^{-8} to 10^{-4} in 0.9% NaCl solution). When scavenger agents were studied on PGE_2 formation, 0.1 mL 10^{-3} M DL-α-tocopherol, deferoxamine mesylate, D-penicillamine, U74500A, and IN were added to the test tube with 10^{-8} M Hb. 10^{-8} M Hb was preferred because this concentration was always able to induce an increase in PGE_2 formation.

All reaction mixtures were brought to 1 mL final volume with the respective incubation buffers.

Slice Incubation [1]

Cerebral cortices from normal and IN-treated rats, dipped in an oxygenated (95% O_2, 5% CO_2) Krebs-Ringer solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$ $7H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 0.1% glucose) at 0°C without or with 10^{-5} M IN, respectively, were sliced to obtain 1–2 mm^3 slices. Each slice was immediately weighed (10–15 mg), washed, and placed in 1 mL of the above solution. The operation time was never longer than 3 min. The incubation was carried out in closed polypropylene tubes for 1 hr in a shaking bath at 37°C in the presence of rat hemoglobin (10^{-9} to 10^{-5} M final concentration). When PGE_2 evaluation was performed, DL-α-tocopherol, deferoxamine mesylate, U74500A, and IN (10^{-4} M final concentration), respectively were added to test tubes containing 10^{-8} M Hb. At the end of incubation, 0.1 N HCl was added and the mixture (pH 3.5) centrifuged (1000 × *g*, 10 min 0°C).

Tests without Hb during both the homogenate and slice procedure were always carried out as control.

PGE_2 Evaluation in Homogenate

After incubation, all homogenates were adjusted to a final pH of 3.5 with 4 mL of citrate buffer (0.2 M citric acid, 0.2 M sodium citrate, 0.1 M EDTA, 10^{-5} M IN, and 2.5% ethanol:pH 3.5) to provide stable conditions for PGE_2 [17]. The mixtures were centrifuged at 0°C for 10 min at 37,000 × *g* and 1.5 mL supernatant solutions were applied to the C18 column (500 mg octadecylsylil silica) previously activated with 3 mL of methanol and then 3 mL of distilled water. After washing with 2 mL of water and 2 mL × 2 of n-hexane, the elution was performed using 2 mL of ethyl acetate. The organic eluates, collected in polypropylene tubes, were evaporated to dryness at room temperature under a stream of Argon. The samples derived from normal or IN-treated rats were dissolved in 3 and 0.3 mL, respectively, of phosphate buffer (0.0255 M NaH_2PO_4, 0.0245 M Na_2HPO_4, 0.01 M EDTA, 0.3% bovine globulin in 0.9% NaCl) pH 6.8. Aliquots (in triplicate of 100 μL) were used for PGE_2 radioimmunoassay using the NEK020 kit provided by New England Nuclear (NEN Chemicals GmbH Dreiech, Germany). The radioactive label was ^{125}I (PGE_2 ^{125}I:specific activity 2200 Ci/mmole) and PGE_2 antiserum showed a cross-reactivity of <2.5 with other PGs. Radioactivity was measured using a Packard counter.

PGE_2 Evaluation in Slices

1 mL solution from all slice tests was extracted with 2 mL ethylacetate and centrifuged (1000 × *g*, for 10 min, 0°C). 1.5 mL of the organic phase was placed in polypropylene tubes and evaporated to dryness at room temperature by an Argon stream. The dry residue was dissolved in 0.3 mL buffer pH 6.8. Radioimmunoassay was performed as described for the homogenate.

LOOH Evaluation [18]

After incubation, 1 mL of both homogenate mixture or slice solution (both from rats with or without IN) was ex-

tracted using 5 mL $CHCl_3$:MeOH (2:1). All assay solutions were treated with Argon fluxes to exclude oxygen. After centrifugation at 1000 × *g* for 10 min at 0°C, 2 mL organic extract were evaporated to dryness at room temperature under an Argon stream. While still under gas flux, 1 mL of acetic acid-chloroform (3:2) solution was added, followed by 50 μL KI (1.2 g/mL aqueous solution). The tube was capped, mixed, and placed in the dark for exactly 5 min. 3 mL of cadmium acetate (5 mg/mL aqueous solution) were added, mixed, and centrifuged at 1500 × *g* for 10 min. Upper layer absorbance was read at 353 nm against blanks containing the complete assay mixture minus homogenate. LOOH values were expressed as nmole/100 mg wet weight using a molar extinction coefficient of 2.8×10^4 M^{-1}.

TBARS Evaluation [19]

After incubation in 0.2 mL homogenate or in 1 mL slice solution (both from rats with or without IN) 0.5 mL thiobarbituric acid (1% w/v in 0.05 M NaOH) were added, followed by 0.5 mL 25% (v/v) HCl. The reaction mixtures, placed in test tubes and sealed with screw caps, were heated in boiling water for 10 min. After cooling, the developed pink chromogen was extracted with 3 mL of n-butanol and then centrifuged at 2000 × *g* for 10 min. The absorbance of supernatant was read at 532 nm. The values were expressed as nmoles MDA/mg protein (MDA/mg prot.), using a standard curve of 1,1,3,3-tetramethoxypropane.

Protein Assay

Protein concentrations were determined according to Lowry *et al.* [20]. Bovine serum albumin was used as standard reference.

Statistical Method

Data are presented as mean values ± SEM. Student's *t*-test (suitably computed by means of the paired or grouped data method) was used, and a probability value of $P < 0.05$ was regarded as significant.

RESULTS

Hb Effect on the Brain Cortex Homogenates

Changes in PGE_2 and LOOH formation occurred when corticocerebral homogenates of normal and IN-treated rats were incubated with different concentrations of Hb (Figs. 1A,B; 2A,B).

The levels of PGE_2 found in normal rats were approximately 10-fold those evaluated in IN-treated rats but, in both cases, the production trend of the PG in response to Hb was similar. PGE_2 value showed a significant increase at 10^{-8} M Hb and a significant decrease at higher concentrations when compared to homogenate without Hb (Figs. 1A, 2A).

When compounds with scavenging and/or chelating

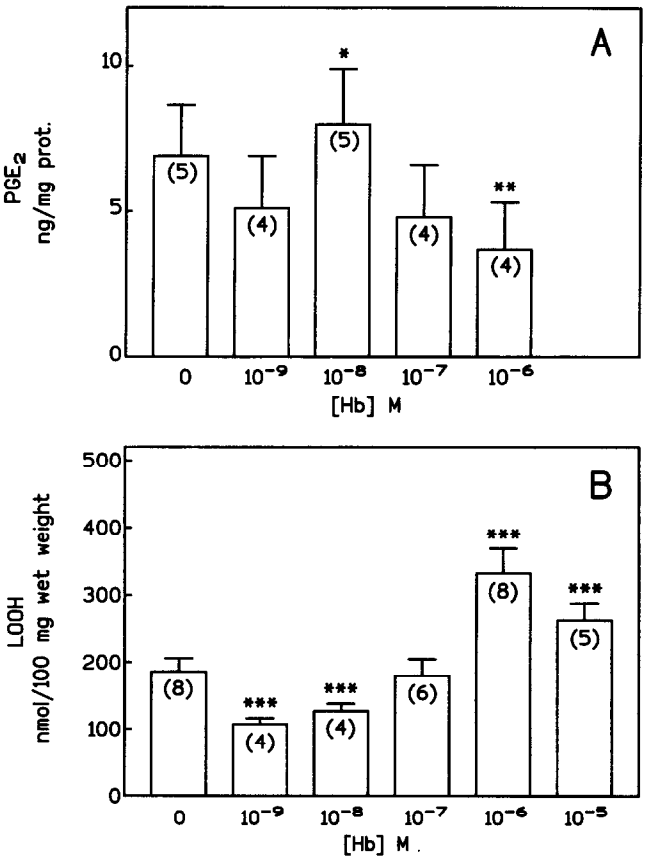

FIG. 1. PGE_2 (A) and LOOH (B) formation in corticocerebral homogenate of normal rats after incubation in the presence of different concentrations of hemoglobin. Each bar represents mean ± SEM. *$P < 0.02$; **$P < 0.005$; ***$P < 0.001$ vs. control. In parentheses, the number of experiments. PGE_2 and LOOH values from nonincubated samples were 4 ± 1.8 ng/mg prot. and 69.5 ± 1.7 nmol/100 mg wet weight, respectively.

properties were added to brain homogenates of both normal and IN-treated animals and incubated in the presence of 10^{-8} M Hb, the PGE_2 levels were significantly lower than those of the respective controls (Table 1). Moreover, these levels were also significantly lower than those found in homogenates without the addition of Hb (normal rats: 6.9 ± 1.8 ng/mg prot.; IN-treated rats: 692 ± 68 pg/mg prot.).

When 10^{-4} M IN was added to IN-treated rat corticocerebral homogenate and incubated in the absence of Hb or in the presence of 10^{-8} M Hb, PGE_2 formation (700 ± 85 and 918 ± 101 pg/mg prot., respectively) was not significantly different from the control (692 ± 68 and 892 ± 85 pg/mg prot. respectively), suggesting that the *in vivo* pretreatment with IN was efficient in inhibiting PES to a maximal extent.

The LOOH levels normal rats were significantly decreased at 10^{-9}–10^{-8} M, but increased at 10^{-6}–10^{-5} M Hb compared to the control (Fig. 1B). However, at all concentrations, the values were significantly ($P < 0.01$ Student's *t*-test; grouped data method) higher than those evaluated in IN-treated rat (Fig. 1B; 2B). The latter values were always

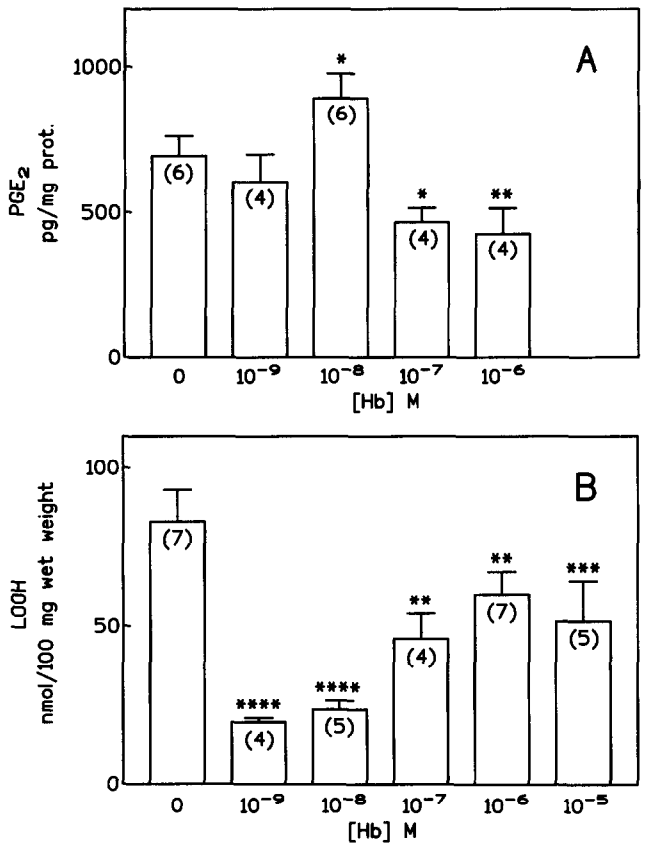

FIG. 2. PGE_2 (A) and LOOH (B) formation in corticocerebral homogenate of IN-treated rats after incubation in the presence of different concentrations of hemoglobin. Each bar represents mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; ****$P < 0.001$ vs. control. In parentheses, the number of experiments. PGE_2 and LOOH values from nonincubated samples were 657 ± 38 pg/mg prot. and 11.6 ± 1.3 nmol/100 mg wet weight, respectively.

significantly lower than the control at all tested Hb concentrations; moreover, we observed a significant difference ($P < 0.001$) between the LOOH levels found at 10^{-8} and 10^{-7} M Hb (Fig. 2B).

TBARS formation did not present quantitative variations (Student's *t*-test: grouped data method) between normal and IN-treated animal groups, but both groups did attain a significant ($P < 0.02$) increase in comparison to controls (3.9 ± 0.5 and 3.6 ± 0.3 nmole MDA/mg prot., respectively) when the incubation was performed in the presence of 10^{-5} M Hb (5 ± 0.7 and 4.5 ± 0.4 nmole MDA/mg prot., respectively).

Hb Effect on Brain Cortex Slices

Brain cortex slices from normal or IN-treated rats presented changes in PGE_2 and LOOH formation after incubation with different concentrations of hemoglobin (Figs. 3A,B; 4A,B).

PGE_2 formation in normal rat corticocerebral slices was significantly increased when incubation was performed

TABLE 1. Effect of scavenging and chelating agents on PGE_2 formation in corticocerebral homogenates of normal and IN-treated rats after incubation with 10^{-8} M hemoglobin

Scavenging and chelating agent (10^{-4} M)	PGE_2	
	Normal rats (ng/mg prot.)	IN-treated rats (pg/mg prot.)
Control	8.0 ± 1.9 (5)	892 ± 85 (4)
α-Tocopherol	4.7 ± 1.5* (4)	410 ± 108* (6)
Deferoxamine	3.2 ± 1.2† (4)	283 ± 21§ (5)
D-Penicillamine	4.0 ± 1.6‡ (4)	586 ± 95* (4)
U74500A	3.7 ± 1.8† (5)	–

Each value represents the mean ± SEM. In parentheses, the number of experiments. *$P < 0.05$; †$P < 0.005$, ‡$P < 0.002$, §$P < 0.001$ vs control. PGE_2 values from nonincubated samples were 4 ± 1.8 ng/mg prot. for normal rats and 657 ± 38 pg/mg prot. for IN-treated rats.

with 10^{-8} to 10^{-6} M Hb concentrations (Fig. 3A). The same trend occurred in the IN-treated rat group (Fig. 4A). All PGE_2 values of normal rat brain cortices were approximately 30-fold those of IN-treated rats (Figs. 3A, 4A).

Scavenging or chelating compounds inhibited the PGE_2 increase when added to the cortex slice medium of both

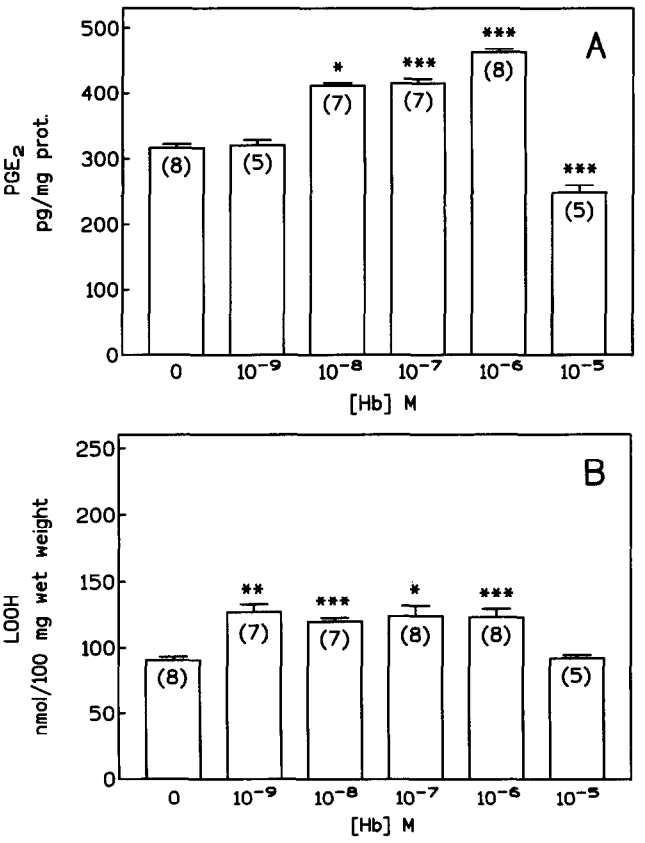

FIG. 3. PGE_2 (A) and LOOH (B) release from brain cortex slices of normal rats after incubation in the presence of different concentrations of hemoglobin. Each bar represents mean ± SEM. *$P < 0.02$; **$P < 0.01$; ***$P < 0.001$ vs control. In parentheses, the number of experiments.

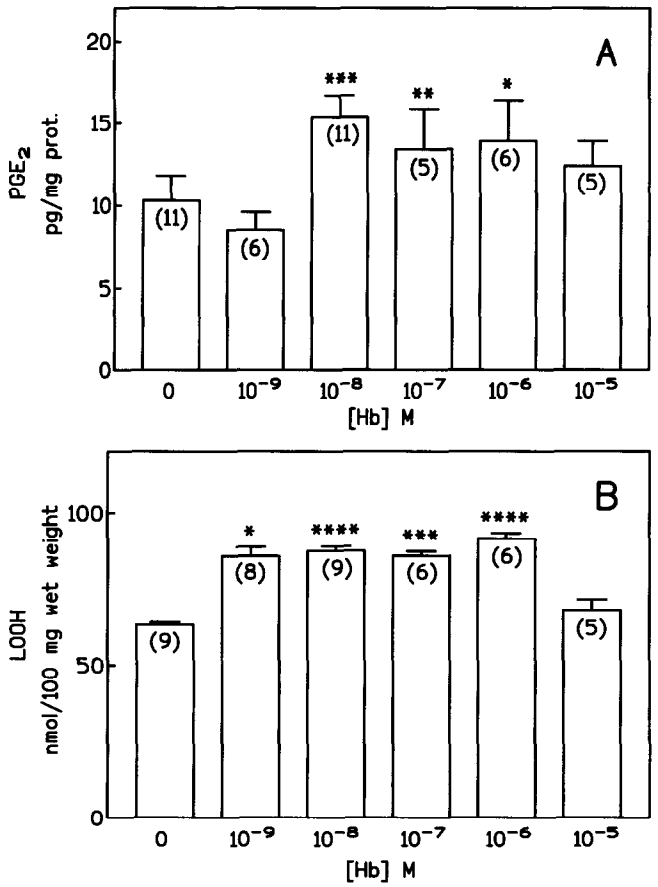

FIG. 4. PGE_2 (A) and LOOH (B) release from brain cortex slices of IN-treated rats after incubation in the presence of different concentrations of hemoglobin. Each bar represents mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; ****$P < 0.001$ vs control. In parentheses, the number of experiments.

normal and IN-treated rats and incubated in the presence of 10^{-8} M Hb (Table 2). Moreover, tocopherol and deferoxamine significantly ($P < 0.02$) decreased PGE_2 formation in comparison to tests without Hb addition (normal rat: 316.4 ± 5.2 pg/mg prot.; IN-treated rat: 10.3 ± 1.5 pg/mg prot.). When IN-treated rat brain cortex slices without Hb or with 10^{-8} M Hb in the reaction medium were incubated with a further presence of 10^{-4} M IN, the PGE_2 values (11.2 ± 1.8 and 14.1 ± 1.3 pg/mg prot., respectively) were not significantly different from that in the controls (10.3 ± 1.5 and 15.4 ± 1.3 pg/mg prot., respectively).

TABLE 2. Effect of scavenging and chelating agents on PGE_2 formation in corticocerebral slices of normal and IN-treated rats after incubation with 10^{-8} M hemoglobin

Scavenging and chelating agent (10^{-4} M)	PGE_2	
	Normal rats (ng/mg prot.)	IN-treated rats (pg/mg prot.)
Control	462 ± 12 (4)	15.4 ± 1.3 (5)
α-Tocopherol	236 ± 4† (6)	7.5 ± 1.2* (4)
Deferoxamine	218 ± 37† (5)	3.7 ± 0.9‡ (4)
U74500A	301 ± 32† (4)	10.4 ± 0.8* (4)

Each value represents the mean ± SEM. In parentheses, the number of experiments. *$P < 0.05$; †$P < 0.005$, ‡$P < 0.001$ vs control.

LOOH formation in cortex slices of normal animals was significantly ($P < 0.01$ Student's t-test: grouped data method) higher than that of IN-treated rats with all Hb concentrations (Figs. 3B, 4B). Moreover, in both cases (normal and IN-treated rats), significant steady-state LOOH increases (10^{-9} to 10^{-6} M Hb) were present in comparison to respective controls but, at 10^{-5} M Hb, LOOH values were significantly ($P < 0.001$) lower than those evaluated at 10^{-9} to 10^{-6} M Hb (Figs. 3B, 4B).

TBARS levels in cortex slice incubation media with 10^{-5} M Hb (0.504 ± 0.067 and 0.473 ± 0.06 nmole MDA/mg prot. normal and IN-treated rats, respectively) reached significant differences ($P < 0.01$ and $P < 0.005$) in comparison to controls (0.121 ± 0.023 and 0.095 ± 0.021 nmole MDA/mg prot. respectively). No significant differences vs the controls were present when the other Hb concentrations were used, nor were such differences observed between IN-treated and untreated animal groups.

DISCUSSION

The results indicate that the variations in PGE_2 and LOOH formation occurring after incubation in the homogenates and in the slices of corticocerebral tissue obtained from normal or IN-pretreated rats may be ascribed to the activity of Hb.

Both groups show similar trends for PGE_2 or LOOH values when homogenation or slice procedures are used, respectively. In particular, it is remarkable that PGE_2 values are always increased when the Hb concentration is 10^{-8} M, whereas no variation is present at 10^{-9} M Hb.

The significant and constant difference noticed between the PGE_2 experimental data of normal and IN-treated rat groups is ascribable to the inhibition of PES activity by IN. On the other hand, the greater formation of PGE_2 observed in the homogenates in comparison to the slices is probably due to the greater availability of arachidonic acid (derived from membrane destruction) to undergo metabolic transformations.

It is reported that PES activity and consequent PG formation are dependent upon LOOH concentration in the cellular microenvironment: 10^{-8} to 10^{-7} M LOOH stimulates cyclooxygenase activity with prostaglandin formation, whereas at 10^{-7} to 10^{-6} M LOOH peroxidase activity prevails with production of free radical oxygen species and consequent lipid peroxidation [21–23].

It is suitable to note that, just as in corticocerebral homogenates from normal rats, the PGE_2 value shows a peak at 10^{-8} M Hb followed by a decrease at higher Hb concentrations; on the contrary, at the same Hb concentrations, the LOOH value presents an opposite correlation.

Therefore, it may be hypothesized that the iron-heme

oxy-redoxy activity of 10^{-9} and 10^{-8} M Hb (to which the activity of endogenous iron compounds is added) induces a decrease in LOOH concentration (decomposition according to the Fenton reaction), providing, at 10^{-8} M Hb, suitable conditions for a predominance of cyclooxygenase activity and, consequently, for PGE_2 formation. At higher Hb concentrations (10^{-6} M), peroxidase activity is prevalent with a decrease in PGE_2 and an increase in lipid peroxidation [24, 25]. At 10^{-5} M Hb, we observe the formation of end lipid peroxidation products as shown by the LOOH decrease and the significant increase in TBARS.

If we consider the significant steady-state increase PGE_2 (10^{-8}–10^{-6} M Hb) and LOOH (10^{-9}–10^{-6} M Hb) produced during the incubation of corticocerebral slices of normal rats, we may suppose that phospholipase enzymes (initially activated by basal lipid hydroperoxides) stimulate the release of definite amounts of arachidonic acid and of other fatty acids from cerebral cell membranes [26]. These compounds constitute the substratum of the PES and the target of free radical oxygen species produced by Hb in the oxygenated incubation medium [27]. In this case, also, when Hb attains a 10^{-5} M concentration, we observe a decrease in PGE_2 and LOOH formation and a significant increase in TBARS, an index of widespread oxidation.

The PGE_2 inhibition by means of scavenger compounds is an index of free radical presence in the activity of PG synthetase. On the other hand, the same effect obtained in the presence of the iron-chelating deferoxamine shows the importance of the iron-heme oxy-redoxy activity of both Hb and/or PES in the induction of PG synthesis.

Because 10^{-4} M IN (a concentration able to inhibit both COX-1-and COX-2-induced cyclooxygenases; [28–30]), when added to both IN-treated rat homogenate and slice incubations, does not significantly modify PGE_2 formation, we may reasonably suppose that PES activity is fully inhibited and that the PGE_2 increase occurring in the IN-treated rat brain cortex with 10^{-8} M Hb is independent of the enzymatic pathway of arachidonic metabolism.

The autocatalytic autooxidizability of AA (accelerated by hydroperoxide products; [31, 32]) and the oxy-redoxy activity of iron-heme on both the oxygen molecule and lipid hydroperoxide with respective formation of superoxide and lipid alkoxy or peroxyl radical are well known reactions [27, 32, 33]. However, if we bear in mind research carried out on the chemical synthesis of PGs [34, 35] and peroxidase and iron heme activity on pure AA [9, 10] we may hypothesize that the PGE_2 increase found in corticocerebral homogenate and slices is derived from autooxidation or Hb-induced oxidation and further peroxy radical cyclization of arachidonic acid.

The mechanism of PGE_2 formation could be hypothesized by considering the reversible reaction $Fe^{3+} + LOO^- \rightleftarrows Fe^{2+} + LOO^{\cdot}$, where the iron oxy-redoxy activity is modulated by the proteic shell of Hb. 10^{-8} M Hb could create a condition whereby the initial reversal would be prevented by AA competing with the lipid peroxyl radical for Fe^{2+}. The oxidation of AA would generate more lipid peroxide, in this case PGG_2, which, in addition to increasing PGE_2, could produce the reverse reaction [36].

At higher Hb concentration, LOOH formation and widespread lipid peroxidation prevail, as shown by the PGE_2 decrease and, finally, by TBARS produced in the presence of 10^{-5} M Hb.

Also, the LOOH decrease, occurring in the presence of 10^{-9} and 10^{-8} M Hb, is probably due to its decomposition, as we have hypothesized for the normal rat. On the other hand, the persistent and significant LOOH decrease in IN-treated rats, also at higher Hb concentrations in comparison to controls, is probably ascribable to the inhibition of cyclooxygenase activity.

As regards PGE_2 and LOOH formation in the incubation medium of IN-treated slices, we can suppose (as in normal rats) that arachidonic acid and other fatty acids are released from brain tissues owing to phospholipase enzyme activation. The prostanoid formation and lipid peroxidation occur because of the presence of Hb-supported reactive oxygen species. The involvement of free radical species and oxy-redoxy activity of Hb are verified by the results obtained when a scavenger such as tocopherol, or a metal-chelating compound such as deferoxamine, or mixed-function compounds such as 21-aminosteroid or penicillamine are used.

On the basis of the above discussion, we may hypothesize that *in vivo* during subarachnoid hemorrhage or ischemia and reperfusion, the Hb released in the cerebral cortex from extravased blood must also attain a 10^{-8} M concentration in the reaction cellular microenvironment to stimulate further PGE_2 production. Moreover, we think that, in such a process, an evaluable part of PG increases may be independent of the enzymatic metabolic pathway of arachidonic acid and directly ascribable to the iron-heme oxy-redoxy activity of Hb.

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