Effect of High-Dose Methylprednisolone and U74006F on Eicosanoid Synthesis After Subarachnoid Hemorrhage in Rats

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Free radicals and lipid peroxidation of membrane fatty acids are thought to play a role in the pathogenesis of arterial vasospasm and the physiopathologic patterns of neuronal damage after subarachnoid hemorrhage. We have evaluated the effects of treatment with either high-dose methylprednisolone every 8 hours or a single dose of U74006F on the temporal profile of ex vivo synthesis of four selected eicosanoids in brain slices after experimental induction of subarachnoid hemorrhage in rats. Prostaglandins D\textsubscript{2} and E\textsubscript{2}, prostacyclin, and leukotriene C\textsubscript{4} levels were determined by radioimmunoassay after 1-hour incubation of the brain slices. The synthesis of prostaglandin D\textsubscript{2} and 6-ketoprostaglandin F\textsubscript{1α} at 48 hours after subarachnoid hemorrhage was significantly higher when compared to sham-operated animals (p=0.01); prostaglandin E\textsubscript{2} release was significantly enhanced at 6 hours after subarachnoid hemorrhage (p=0.01). The release of the lipoxgenase metabolite was significantly enhanced at 1, 6, and 48 hours after subarachnoid hemorrhage induction. Both treatment regimens significantly reduced the ex vivo synthesis of prostaglandin D\textsubscript{2}, prostaglandin E\textsubscript{2}, and leukotriene C\textsubscript{4} at 1, 6, and 48 hours after subarachnoid hemorrhage, whereas the effects on 6-ketoprostaglandin F\textsubscript{1α} synthesis differed in the two treatment groups. U74006F enhanced the synthesis of prostacyclin metabolite in the early phase after subarachnoid hemorrhage, and high-dose methylprednisolone reduced the increasing synthesis at 48 hours. A strict comparison between the two treatments was not possible because of the different modalities of administration. However, these data suggest that the antioxidant effect of single-dose treatment with U74006F influenced the early and delayed effects on enzymatic lipid peroxidation, whereas the effects of methylprednisolone administration every 8 hours were more significant in the delayed phase. (Stroke 1991;22:215–220)
investigate 1) whether the antioxidant activity of U74006F could influence arachidonic acid synthesis and 2) whether the effect of a single administration of U74006F is similar to that exerted after repeated methylprednisolone administration. We stress that the widely accepted treatment with methylprednisolone was used not with the intention of making a strict comparison to the aminosteroid modality of action, but to collect standardized control data to correlate with the effect of single-dose administration of U74006F.

Materials and Methods

We conducted experiments on male Sprague-Dawley rats (Charles River strain, Calco, Como, Italy) weighing 375-425 g, using the experimental SAH procedure according to previously reported studies.24-26 General anesthesia was induced with 3% halothane (70%: 30%, N2O: O2) and maintained with 0.75% halothane in the gas mixture. A burr hole was made at the interparietal/occipital suture connection using a refrigerated twist-drill, and a small catheter (model PE-10, Clay-Adams, Parsippany, N.J.) was inserted into the cisterna magna. A femoral artery was cannulated for anerobic sampling of blood to measure pH, Pco2, and Po2, and arterial blood pressure was monitored with an indirect blood pressure sensor (model 446420, Bel-Art Products, Pequannock, N.J.). Body temperature, monitored by a rectal thermometer, was adjusted and maintained near 37°C by external heating. When the rats were in a steady respiratory state with arterial Po2 and PCO2 near 110 and 30-40 mm Hg, respectively, 0.35 ml autologous arterial blood was collected from the femoral artery, and an aliquot of 0.30 ml was injected into the cisterna magna via the catheter within approximately 2 minutes. Before SAH induction, a sample of cerebrospinal fluid of approximately 0.01-0.03 ml was gently drawn to limit modifications in intracranial pressure. The animals were held in a 20° head-down position.

Animals were divided into the following four experimental groups of seven animals each: 1) sham-operated rats submitted to surgical procedure and injected with 0.30 ml saline at 37°C, 2) SAH rats subjected to SAH procedure with injection of 0.30 ml autologous arterial blood, 3) SAH rats treated with 3 mg/kg i.v. U74006F within 10-15 minutes after SAH induction, and 4) SAH rats treated with 30 mg/kg i.v. methylprednisolone within 10-15 minutes after surgical procedure, and 15 mg/kg i.v. every 8 hours for the rats killed at 48 hours. Biochemical evaluations were performed at 1, 6, and 48 hours after the experimental SAH procedure. The rats were decapitated and the brains were carefully removed and immediately frozen in dry ice and maintained in a prerefrigerated glove box at -22°C. Cortex slices, measuring approximately 1 mm², were bound-cut, weighed (10-15 mg), and placed immediately in 1 ml oxygenated 95% O2-5% CO2 Krebs' solution, pH 7.4, containing (mM) NaCl 118, KCl 4.7, MgSO4 7, H2O 1.2, KH2PO4 1.2, NaHCO3 25, and glucose.

The interval between decapitation and the beginning of incubation never was longer than 3 minutes. The tubes containing the medium and slices were closed, and the slices were incubated at 37°C in a shaking water bath for up to 1 hour. After this procedure, the medium was decanted and centrifuged in a refrigerated centrifuge at 3,000 rpm at 0°C. Three aliquots of the supernatant were kept at -80°C until analysis.

We determined levels of arachidonic acid metabolites using the radioimmunoassay technique for prostaglandin D2 previously described in detail.5,6 Radioimmunoassay kits for prostaglandin E2 (NEK-020) and 6-ketoprostaglandin F1α (NEK-008) were provided by New England Nuclear (NEN) Chemicals GmbH, Dreieich, FRG. The radioactive labels were 125I for prostaglandin E2 and 3H for 6-ketoprostaglandin F1α. Antiserum for these metabolites had less than 2.5% of cross-reactivity with other prostaglandins. Immunoreactive leukotriene C4-like activity was detected with the radioimmunoassay technique, according to Levine et al,27 using an antiserum (NEN Chemicals) to leukotriene C4 that has a cross-reactivity of 10.1% with leukotriene D4, 2.3% with leukotriene E4, 0.07% with hydroxyeicosatetraenoic acid, and 0.006% with leukotriene B4. Ten milliliters Atomlight High Sample Capacity Scintillation Solution (NEF-968) was added to each sample (supplied by United Technologies Packard, Packard Instruments, Downers Grove, III.). Radioactivity was measured using a liquid scintillation spectrometer (model 3220, Packard Instruments) as previously described.28

Results are expressed in picograms per milligram of protein. The protein content of homogenate was assayed according to Lowry et al29 with serum albumin as a standard. The assay sensitivity was 15 pg/mg protein. Statistical analysis was performed using analysis of variance and Tukey's test for multiple comparisons. Statistical significance was accepted for values of p<0.05.

Results

Figure 1 represents the values of prostaglandin D2 ex vivo release after the experimental procedure. The release was enhanced after the hemorrhage, and at 48 hours after SAH was significantly higher when compared to values from sham-operated animals (p<0.01). High-dose methylprednisolone significantly decreased the release at 48 hours, and the values in the treated group did not differ from those in the sham-operated group. U74006F significantly reduced the late peak of prostaglandin D2 release (p<0.05), even though this effect was significantly lower when compared to animals treated with high-dose methylprednisolone.

Figure 2 shows a significant enhancement of prostaglandin E2 release at 6 hours after the SAH procedure (p<0.01). A significant reduction in prostaglandin E2 release at 6 hours after U74006F and high-dose methylprednisolone administration was observed. In animals treated with U74006F, a signif-
significant conflicting increase in prostaglandin E₂ synthesis and release was observed at 48 hours.

The release of prostacyclin was significantly enhanced at 48 hours after SAH induction. High-dose methylprednisolone significantly reduced the release at 48 hours (Figure 3). A trend toward an inhibitory effect of U74006F at 48 hours and of methylprednisolone at 6 hours was evident, although statistical significance was not achieved because of the small number of animals used in each group and the wide range of the data. More experiments are ongoing to verify the effects on eicosanoid synthesis at 3 and 4 days after administration of U74006F and high-dose methylprednisolone.

The release of the lipoxygenase metabolite was dramatically enhanced at 1, 6, and 48 hours after SAH induction, whereas high-dose methylprednisolone significantly decreased the release at 1, 6, and 48 hours. The inhibitory effect on leukotriene C₄ synthesis capacity is characteristic and showed a progressive inhibitory trend, with greater significance in the late phase (Figure 4). On the other hand, in the early posthemorrhagic period, lipoxygenase activation was reduced by U74006F, with the effect more pronounced at 1 and 6 hours (p<0.01).

Discussion

In a previous study, we showed enhanced activity of both cyclooxygenase and lipoxygenase pathways in the cerebral cortex of rats after experimental SAH. Similar findings were reported for the vascular compartment and the subarachnoid space. The ex vivo method provides information about the residual capacity of brain tissue to synthesize arachidonic acid metabolites after a pathological event, as previously discussed. The aim of the present study was to compare the efficacy of a single dose of a new aminosteroid (U74006F) with routine standard treat-
ment with high-dose methylprednisolone, rather than a pharmacologic comparison of the effects and mechanisms exerted on eicosanoid synthesis. Thus, we administered a single dose of U74006F to verify either its antioxidant properties or a delayed effect on enzymatic lipid peroxidation, i.e., on cyclooxygenase and lipoxygenase pathways. Methylprednisolone was administered every 8 hours after the experimental procedure.

The global effect of U74006F on arachidonic acid metabolism was mostly evident during the early phase (1–6 hours) after SAH. On the other hand, the effect of high-dose methylprednisolone was evident on both metabolic pathways and was more pronounced during the late phase, 48 hours after SAH. Looking at the antioxidant properties of U74006F, the significant effect of an early single injection on the late-phase synthesis of leukotriene $C_4$ seems more intriguing, although no mechanistic conclusion can be drawn about the specific mechanism.

In a recent review, Braughler and Hall stressed that lipid peroxidation, phospholipase activation, and fatty acid release are inextricably related. Superoxide radicals are intermediate products of arachidonic acid metabolism, in both cyclooxygenase and lipoxygenase pathways. The resulting lipid peroxidation may enhance leukotriene synthesis in a chain-branched reaction.

The available data suggested that methylprednisolone acts on a glucocorticoid receptor, stimulating the synthesis of lipocortin protein, which inhibits phospholipase A2 and the release of arachidonic acid. High-dose methylprednisolone also inhibits the hydrolysis of membrane phospholipids through a receptor-independent antioxidant mechanism.

Our results confirm this hypothesis previously suggested.

**Figure 3.** Ex vivo release of 6-ketoprostaglandin $F_{1\alpha}$ (pg/mg protein; mean±SEM) in rats subjected to experimental subarachnoid hemorrhage (SAH), treated with the 21-aminosteroid U74006F, and treated with high-dose methylprednisolone (MP). SAH vs. sham-operated (SHAM-OP.): **p<0.01; SAH-MP vs. SAH: !p<0.05.

**Figure 4.** Ex vivo release of leukotriene $C_4$ (pg/mg protein; mean±SEM) in rats subjected to experimental subarachnoid hemorrhage (SAH), treated with the 21-aminosteroid U74006F, and treated with high-dose methylprednisolone (MP). SAH vs. sham-operated (SHAM-OP.): *p<0.05; **p<0.02; ***p<0.01; SAH-U74006F vs. SAH: **p<0.02; ***p<0.01; SAH-MP vs. SAH: !p<0.05; !p<0.02; !p<0.01.
suggested by Hall and coworkers and answer the question of whether U74006F could indirectly inhibit leukotriene release.

Glutathione peroxidase acts on lipid hydroperoxides and has been shown to inhibit lipoxygenase. Our results point out the early effect of U74006F on leukotriene C4 release at 1 and 6 hours and suggest that the aminosteroid, besides the specific antiperoxidative activity, may also influence brain synthesis of eicosanoids after SAH.

Nevertheless, the pharmacologic effects of these two drugs should also be evaluated, looking at specific pathophysiological aspects of SAH, that is, changes in cerebral blood flow and vasospasm. High-dose methylprednisolone was recently shown to improve cerebral blood flow without a concomitant increase of intracranial pressure after experimental SAH in cats. This suggests a direct effect of this steroid on microcirculatory regulation, which would modulate the production of vasoactive compounds, with special regard to eicosanoids. Our results further support this hypothesis by demonstrating a significant effect of high-dose methylprednisolone on both lipoxygenase and cyclooxygenase pathways and on the biosynthesis of vasoactive eicosanoids.

The role played by arachidonate metabolites in the pathogenesis of arterial vasospasm after SAH has been widely discussed. Prostaglandin D2 and leukotriene C4 have well-documented vasoconstrictive activity on cerebral arteries, with high levels also detected in the cisternal cerebrospinal fluid of patients with vasospasm after the aneurysm rupture. In the same experimental model of SAH, a biphasic pattern of arterial spasm was shown: an acute phase occurring 10–15 minutes after SAH, followed by a late phase up to 48 hours after SAH, which is more important from a metabolic point of view.

U74006F was shown to reduce the postischemic hypoperfusion of brain tissue; this effect may be related to its protective effect on cerebral microvasculature and to an inhibition of lipid peroxidation. The results of the present study suggest that U74006F may exert a significant inhibitory effect on the lipoxygenase pathway and reduce the release of spasmodenic substances such as leukotriene C4, whereas its effect on the synthesis of the vasodilating agent prostacyclin did not seem so dramatic. On the other hand, high-dose methylprednisolone significantly reduces the severity of the typical degenerative arteriopathy of the delayed vasospasm. As shown in the present study, this could be partially related to a significant inhibitory effect on brain eicosanoid synthesis in the late phase.

In conclusion, our results show significant changes of the ex vivo synthesis of eicosanoids from cerebral cortex after experimental SAH, indirectly stressing the involvement of oxygen free radicals and lipid peroxidation in brain response to hemorrhage. Moreover, they suggest a possible double protective mechanism for U74006F. In the early phase after SAH, the inhibitory effect on iron-catalyzed lipid peroxidation influences eicosanoid synthesis; in the late phase, a reduced synthesis of vasoconstrictive eicosanoids, such as leukotriene C4 and prostaglandin D2, could be effective in reducing or preventing vasospasm.

High-dose methylprednisolone exerts a receptor-dependent effect on phospholipase A2 and inhibits both cyclooxygenase and lipoxygenase pathways. This effect is more evident in the late phase of SAH. Both treatments could act in a complementary way to provide brain protection after hemorrhage.

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