Which is more effective in reducing secondary brain damage resulting from cyclooxygenase expression following traumatic brain injury: calcium channel blockers or cox inhibitors?

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ABSTRACT

الأهداف: تقييم مواقع (COX–1) و (COX–2) عقب التعرض لإصابة الدماغ (TBI)، وآثار العاملان العلاجيان ما إذا كانا يقدمان أو لا يقدمان تثبيطاً لخلايا (COX).

الطريقة: في هذه الدراسة تم استعمال 40 أرنباً لإجراء نموذج إصابة الدماغ (TBI)، وقسمة إلى أربعة مجموعات (عدد=10) في كل مجموعة. أجريت هذه الدراسة بكلية الطب بجامعة أفيون كوساتيب التعليمية – تركيا، في يونيو 2004م. تم تحليل ملفات ظهور (COX–1) الخلوي المميز وبروتين (COX–2) عقب التعرض لإصابة الدماغ (TBI)، وتم تقييم آثار العاملين العلاجيين اندوميثاسين ونيموديبين على تثبيط خلايا (COX) بالكيمياء النسيجية المناعية.

النتائج: كشفت الدراسة أن ظهور (COX–1) و (COX–2) قد ازداد بشكل ملحوظ في التهاب البطانة الوعائية، وخلايا العضلة الناعمة و(CD68+ microglia/macrophages) عقب التعرض لإصابة الدماغ (TBI). في نتائجنا، ثبط اندوميثاسين ظهور خلايا (COX) الدبقية أكثر من نيموديبين، ولكن كلاهما لم يؤثر على ظهور (COX–1) في البطانة و ظهور (COX–2).

خاتمة: التراكم المحصور لعنصر (COX–1) عند منطقة الآفة تشير إلى استجابة الالتهاب الحاد ودور (COX–1) في إصابة الدماغ (TBI). كشفت هذه الدراسة أنه يجب على ظهور (COX–1) أن يكون هدفاً دوائياً عقب التعرض لإصابة الدماغ (TBI)، كما يجب أن يتم تقييم (COX–2) في هذا الجانب. ويعتبر اندوميثاسين أكثر فعالية من نيموديبين لسد (COX–1).

Objectives: To evaluate localizations of cyclooxygenase (COX)-1 and COX-2 following traumatic brain injury (TBI) and the effects of 2 therapeutic agents on COX inhibition.

Methods: Forty rabbits were used in this study for developing a TBI model and divided into 4 groups (n=10) at Afyon Kocatepe University School of Medicine, Afyonkarahisar, Turkey in June 2004. Differential cellular COX-1 and COX-2 protein expression profiles were analyzed following TBI, and the effects of 2 therapeutic agents, indomethacin and nimodipine, on COX inhibition were evaluated immunohistochemically.

Results: This study revealed that COX-1 and COX-2 protein expression were significantly increased in vascular endothelial, smooth muscle cells, and CD68+ microglia/macrophages following TBI. Indomethacin inhibited the COX expression in glial cells more than nimodipine, however, both did not affect endothelial COX-1 and COX-2 expression.

Conclusion: The restricted accumulation of COX-1 at the perilesional area points to an acute inflammatory response and the role of COX-1 in TBI. This study revealed that COX-1 expression should be a pharmacological target following TBI, and COX-2 should also be evaluated in this aspect, and indomethacin is more effective than nimodipine for blocking COX-1.

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Secondary brain damage depends on several complex pathophysiological events that evolve over time and place.[1,2] The intracellular influx of seconder messenger calcium (Ca^{++}) activates the phospholipase A2 and cyclooxygenase (COX) following traumatic brain injury (TBI). The ensuing lipid peroxidation causes

cell membrane injury and induces the release of toxic prostanoids. This process is supported by reactive oxygen species (ROS) production. The ROS activates endogenous scavenging mechanisms and triggers the scavenging process characterized by apoptosis and inflammation.[3-5] Prostanoids are the major contributors of the intrinsic inflammatory response of the CNS (such as prostaglandin [PG]E_2, PGD_2, $PGF_{2\alpha}$, thromboxane A_2, PGI_2). These are synthesized by a prostaglandin endoperoxide synthase (known as COX), which is a rate limiting step in this process.[3,6,7] Furthermore, COX has also peroxidase activity and can cause free oxygen radical production and dopamine quinones independent of its mechanism of arachidonic acid.[8] There are several isoforms of COX: expressed COX-1, rapidly inducible COX-2, and the hypothetically proposed isoform of COX-3.[6,9] Although there are some significant differences in structure and gene regulations of COX-1 and COX-2, significant similarities of protein structure and enzymatic functions are observed.[10-13] The metabolites of COX-2 activated by TBI are major neurotoxic mediators.[3,14-16] The enzymatic function of activated microglia and the likewise proinflammatory COX-1 following TBI remains unknown.[6,17,18] In the present study, to provide the pathophysiological role of COX-1 in rabbit TBI, we analyzed the COX-1 and COX-2 expression in brain injured rabbits and unaltered control brains. We also analyzed the effect of 2 therapeutic agents (indomethacin and nimodipine) on this process. Indomethacin is a non-specific COX inhibitor and may affect both COX-1 and COX-2, and nimodipine affects Ca^{++} channels and may decrease the production and extravasation of COX type substances synthesized from arachidonic acid.

Methods. After obtaining ethical approval for the study from the Animal Ethics Committee of Afyon Kocatepe University, the experiment was carried out with 40, 3-month old domestic race and mixed gender rabbits weighting between 450-500 gr from a special rabbit growth center in Istanbul. The animals were randomly divided into 4 groups (n=10) as follows: 1) sham, 2) trauma group without drug, 3) trauma group with nimodipine, 4) trauma group with indomethacin. All the animals' respiratory and heart rates, temperature and 0_2 levels were monitored. The animals in the sham group were anesthetized with midazolam 2 mg/kg, and cerebral tissue samples were taken out without any previous head injury. Tissue samples were placed into formalin solution and transferred to Afyon Kocatepe University School of Medicine Pathology Department in June 2004. The animals in group 2 were also anesthetized with midazolam 2 mg/kg and traumatized with 300 gm weight falling from one meter height. They were sacrificed 120 minutes after the trauma and cerebral tissue samples were obtained. If an animal died before 120 minutes, it was excluded from the study. In group 3, following the same anesthesia and trauma model, nimodipine was given at a dose of 0.02 mg via the right jugular vein 30 minutes after the trauma model. Cerebral tissue samples were taken 120 minutes following the trauma. In group 4, following the same anesthesia and trauma model, one mg indomethacin was given thorough a previously placed orogastric feeding catheter 15 minutes after the trauma and the animal were decapitated and cerebral tissue samples were taken at 120 minutes after trauma. At the injury site the cortex of the brain had a red-purple color and sometimes there was dural injury and arachnoid tears, and the injury site was changeable, namely, right or left parietal hemisphere. The cerebral tissue samples of the animals were put into 10% formalin solution for 24 hours and evaluated macroscopically, and prepared according to paraffin embedding routine tissue preparation technique. Tissue samples were taken from lesions and neighboring areas (2-5 sample from each animal, mean 3). Following this, paraffin blocks of approximately 4 µm in thickness were cut from each of the tissue samples The cut sections were stained with Hematoxylin-Eosin and evaluated with light microscope to determine the tissue blocks suitable for immunohistochemical staining. The 4 µm thick sections were prepared from the previously selected blocks and placed into slides covered with poly-L-lysin. The double staining (immunohistochemical) procedure performed to sections placed into poly-L-lysin covered slides were arranged as follows: They were boiled 2 times for 10 minutes in citrate buffer (pH 6) microwave oven and washed with tris buffer and then incubated with 1% H_2O_2 for 5 minutes, and then washed with tris buffer. They were then incubated with glial fibrillary acidic protein (GFAP) (prediluted, Neomarkers, USA) for glial cells, with Factor VIII antibodies for vessels for 30 minutes and washed with tris buffer. All were incubated with biotinylated horseradish peroxidase solution (DAKO, Denmark) for 45 minutes and washed with tris buffer. The staining intensity was controlled in diaminobenzidine solution as chromogen and kept for 5-15 minutes and washed with distilled water. The samples were kept in double stain block solution (DAKO, Denmark) for 3 minutes and washed with tris buffer. Then they were incubated with anti-COX-1 (1:50 dilution, Santa Cruz, USA) or anti-COX-2 (1:50 dilution, Santa Cruz, USA) antibodies at 4ºC for one night and washed with tris buffer. After this procedure the samples were incubated in alkaline phosphatase-conjugated avidin-biotin complex for 45 minutes and washed with tris buffer.

Staining intensity was controlled in fast red chromogen solution and kept for 5-15 minutes and washed with distilled water. The samples were counterstained with Mayer's Hematoxylin for 10 minutes and washed with distilled water. All of them were dried and fitted for evaluation. The stained slices were viewed under a light microscope. The immunohistochemistry was performed for analysis of COX-1 and COX-2 expression at glial cells and vessels separately. The number of GFAP(+) cells presenting COX-1 and COX-2 expression was counted at 10 different fields with 400X for every slide. The sum of the positive stained cells was calculated in 10 fields for every case. The total number of positive stained cells in every study group was determined and divided into the number in that group, so that the group mean was achieved. The positive staining in vessels was determined with factor VIII. The presence of COX-1 and COX-2 antibodies was also determined by the double staining method. The COX-1 and COX-2 expression in endothelial cells of vessels stained with factor VIII were accepted as positive for the presence of COX-1 and COX-2. For every slide, using 400 magnifying power, 10 different fields were evaluated and the vessels expressing COX-1 and COX-2 were counted. Following that analysis the total number of positive stained vessels in every 10 different fields was determined. The sum of positive stained vessels in each group was divided into the number of cases, and the mean value was obtained. For every group, the mean values of COX-1 and COX-2 expression in factor VIII stained vessels of GFAP stained cells were compared statistically.

Using SPSS 10.0, the results were evaluated with variance analysis, and ANOVA. The difference between the groups was evaluated using DUNCAN analysis. A p-value ≤ 0.05 was considered statistically significant.

Results. In the control group, there was no observed neuropathological alteration, except for a few microglia and macrophage cells. The mean glial COX-1 expression was 2.6 ± 0.6 (mean ± SEM) in the control group, and 27.7 ± 3.52 in group 2 (trauma without treatment), indicating that TBI altered the expression of COX-1 and elevated it significantly in endothelial, smooth muscle, and microglia/macrophage cells. The elevation observed in COX-1 expression following trauma was statistically significant (p=0.035). The mean value of group 3 (nimodipine treated trauma group) was 14 ± 2.36, significantly different to group 2 (p=0.045). However, the results of this group revealed that nimodipine did not affect glial cells. The mean value of COX-1 expression in the indomethacin treated trauma group (group 4) was 7.7 ± 2.28. This result showed that indomethacin inhibited COX expression in glial cells. Although the value was higher numerically than that of the control group, this difference was not statistically significant. Our present study also showed that the mean value of COX-1 expression was 3.2 ± 0.71 in the control group. The mean value of COX-1 expression was 26.7 ± 2.22 in group 2, and the difference between the control and group 2 was statistically different (p=0.038). The mean values were 11.7 ± 1.79 in group 3, and 13 ± 0.85 in group 4. Although the difference between control and drug-treated groups was statistically different, there was no difference between the drug-treated groups. This result indicates that both agents did not affect endothelial COX-1 expression. The results of glial COX-2 expression revealed that the mean values were 0.6 ± 0.43 for the control group, 3.8 ± 0.92 for group 2, 2.7 ± 0.21 for group 3, and 1.8 ± 0.77 for group 4, indicating that the value of the indomethacin treated group did not differ from the control group, with significant COX-2 blockage in glial cells. There was no statistically significant difference between group 2 and group 3, indicating that nimodipine did not inhibit COX-2 expression. The mean values of endothelial COX-2 expressions were 3.9 ± 0.59 for group 2, 2.5 ± 0.75 for group 3, and 2.6 ± 0.31 for group 4. The comparison of these results revealed no statistical significance, indicating that both drugs did not produce endothelial COX-2 inhibition.

Discussion. Traumatic brain injury increases COX-1+ cells in perivascular and Virchow-Robin space. The perivascular space is a transmission route of extravasated cells in the CNS. During the early period of trauma COX-1$^+$ monocytes intermingle and accumulate within the intima and vessel wall leaving the vessel lumen that further increases gradually. Schwab et al[19] previously reported that early COX-1$^+$ cell accumulation in the Virchow-Robin space paralleling the leukocyte infiltration period and the persistence of COX-1$^+$ cells are 2 important features observed in damaged tissue following trauma.[19] Blood derived COX-1$^+$ cells induce a proinflammatory response after being extravasated and engrafted through the peripheral tissue. This response produces the beginning stage of a more prolonged inflammatory response and secondary tissue injury. Additionally, persistent COX-1$^+$ cell accumulation increases the incidence and prevalence of Alzheimer disease by producing the residual inflammatory risk in patients subjected to head trauma.[5,20,21] The head injury is a serious risk factor for developing Alzheimer's disease and inflammation mediated by trauma stimulates the production of ß-APP. These inflammatory mediators can be detected intracerebrally after trauma.[19,22] Since the

inflammatory mechanisms are considered to be involved in Alzheimer's disease pathogenesis, long-term steroid treatment is recommended for patients with brain injury to prevent Alzheimer's disease following trauma.[22-24] Based on the experimental,[25,26] and clinical studies,[27,28] non-selective COX blockage, including COX-1 might delay the onset or decrease the incidence of Alzheimer's disease. Thus, COX-1 might be assumed as a potential substrate for nonsteroidal antiinflammatory activation following head injury.

There is evidence for the pathophysiological role of post-traumatic COX-1 expression activated by macrophages and microglial cells. Activated macrophages and microglial cells support angiogenesis,[29] and regeneration,[30,31] and also produce toxic agents at the same time.[32] These results indicate that local increase in COX production causes COX-2 expression and simultaneous inflammatory cell accumulation causing COX-1 expression.

The results of the present study revealed that nimodipine prevented post-traumatic oxidative stress, decreased the COX production partially in the endovascular space, even in the short-term.[33-35] However, the most pronounced inhibition was achieved by indomethacin treatment. These results indicate that in a long-term study, both agents could be effective in delaying or preventing the development of chronic disease processes. Additionally, the blockage of glial COX-1 expression by indomethacin was pronounced, and the results of single dose indomethacin treatment were similar to the control group. It may be possible to obtain a more pronounced effect by using repetitive doses or long term indomethacin treatment. There are several reports in the literature demonstrating the effect of indomethacin given early, similar to our study. This may be attributed to its cumulative effect on COX inhibition. We have demonstrated that the Ca++ blocker nimodipine partially decreased the production and extravasation of COX type substances synthesized from arachidonic acid. Therefore, we suppose that the therapeutic administration of indomethacin might demonstrate its effect by decreasing vasospasm.[36]

From our results we can easily see that indomethacin had a statistically significant effect on COX-1 expression on glial cells, and the same effect can be seen at nimodipine, but lower than indomethacin. At the same time, both of the agents had the same effect on endothelial COX-1 expression when compared with the untreated traumatic group. However, when we compared them with controls, there was no significant blockage of COX expression. After evaluating endothelial COX-2 values, there was no statistically significant inhibition of expression, however, indomethacin had a significant effect on blocking glial COX-2 expression. Therefore, both, but especially indomethacin may be valuable in combined treatment at the post-traumatic period. Further studies are needed to confirm the long-term effectiveness of both agents in standard treatment protocols.

In conclusion, indomethacin inhibited COX-1 expression in glial cells, but the nimodipine dose did not have the same effect. Nimodipine has a minimal perivascular effect. Both of the agents have no effect on COX-2 expression.

References

1. Graham DI, Gennarelli TA. Trauma. In: Graham DI, Lantos PL, editors. Greenfield's Neuropathology. 6th ed. London: Arnold; 1997. p. 197-262.
2. Hsu CY, Hu ZY, Doster SK. Cell mediated injury. In: Narayan RK, Wilberger JE, Povlishock JT, editors. Neurotrauma. New York (NY): McGraw-Hill; 1996. p. 1433-1444.
3. Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. ***Trends Neurosci*** 1999; 22: 391-397.
4. Hall ED. Free radicals and lipid peroxidation. In: Narayan RK, Wilberger JE, Povlishock JT, editors. Neurotrauma. New York (NY): McGraw-Hill; 1996. p. 1405-1420.
5. Mayeux R, Ottman R, Tang MX, Noboa-Bauza L, Marder K, Gurland B, et al. Genetic susceptibility and head injury as risk factors for Alzheimer's Disease among community-dwelling elderly persons and their first-degree relatives. ***Ann Neurol*** 1993; 33: 494-501.
6. Kaufmann WE, Andreasson KI, Isakson PC, Worley PF. Cyclooxygenases and central nervous system. ***Prostaglandins*** 1997; 54: 601-624.
7. Mattson MP. Modification of ion homeostasis by lipid peroxidation: roles in neuronal degeneration and adaptive plasticity. ***Trends Neurosci*** 1995; 21: 53-57.
8. Hastings TG. Enzymatic oxidation of dopamine: the role of prostaglandin H synthase. ***J Neurochem*** 1995; 64: 919-924.
9. Willoughby DA, Moore AR, Colville-Nash PR. COX-1, COX-2, and COX-3 and the future treatment of chronic inflammatory disease. ***Lancet*** 2000; 355: 646-648.
10. Crofford LJ. COX-1 and COX-2 tissue expression: implications and predictions. ***J Rheumatol*** 1997; 24: 15-19.
11. Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, et al. Cyclooxygenase in biology and disease. ***FASEB J*** 1998; 12: 1063-1073.
12. Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. ***Annu Rev Biochem*** 2000; 69: 145-182.
13. Williams CS, Dubois RN. Prostaglandin endoperoxide synthase: why two isoforms? ***Am J Physiol*** 1996; 270: G393-G400.
14. Nakayama M, Uchimura K, Zhu RL, Rose M, Stetler RA, Isakson PC, et al. Cyclooxygenase-2 inhibition prevents delayed death of CA1 hippocampal neurons following global ischemia. ***Proc Natl Acad Sci U S A*** 1998; 95: 10954-10959.
15. Nogawa S, Zhang F, Ross ME, Iadecola C. Cyclo-oxygenase-2 gene expression in neurons contributes to ischemic brain damage. ***J Neurosci*** 1997; 17: 2746-2755.
16. Sairanen T, Ristimaki A, Karjalainen-Lindsberg ML, Paetau A, Kaste M, Lindsberg PJ. Cyclooxygenase-2 is induced globally in infarcted human brain. ***Ann Neurol*** 1998; 43: 738-747.
17. Mallat M, Chamak B. Brain macrophages: neurotrophic effector cells? ***J Leukoc Biol*** 1994; 56: 416-422.

18. Smith CJ, Zhang Y, Koboldt CM, Muhammad J, Zweifel BS, Shaffer A, et al. Pharmacological analysis of cyclooxygenase-1 in inflammation. ***Proc Natl Acad Sci U S A*** 1998; 95: 13313-13318.
19. Schwab JM, Beschorner R, Meyerman R, Gözalan F, Schluesener HJ. Persistent accumulation of cyclooxygenase-1-expressing microglial cells and macrophages and transient upregulation by endothelium in human brain injury. ***J Neurosurgery*** 2002; 96: 892-899.
20. Mortimer JA, van Duijn CM, Chandra V, Fratiglioni L, Graves AB, Heyman A, et al. Head trauma as a risk factor for Alzheimer's disease: a collaborative re-analysis of case-control studies. EURODERM Risk Factors Research Group. ***Int J Epidemiol*** 1991; 20 Suppl 2: S28-S35.
21. Plassman BL, Havlik RJ, Steffens DC, Helms MJ, Newman TN, Drosdick D, et al. Documented head injury in early adulthood and risk of Alzheimer's disease and other dementias. ***Neurology*** 2000; 55: 1158-1166.
22. Brugg B, Dubreiul YL, Huber G, Wollman EE, Delhaye-Bouchaud N, Mariani J. Inflammatory processes induce beta-amyloid precursor protein changes in mouse brain. ***Proc Natl Acad Sci U S A*** 1995; 92: 3032-3035.
23. Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, et al. Inflammation and Alzheimer's disease. ***Neurobiol Aging*** 2000; 21: 383-421.
24. Yasojima K, Schwab C, McGeer GE, McGeer PL. Distribution of cyclooxygenase-1 and cyclooxygenase-2 mRNAs and proteins in human brain and peripheral organs. ***Brain Res*** 1999; 830: 226-236.
25. Lim GP, Yang F, Chu T, Chen P, Beech W, Teter B, et al. Ibuprofen suppresses plaque pathology and inflammation in a Mouse model for Alzheimer's Disease. ***J Neurosci*** 2000; 20: 5709-5714.
26. Wyss-Coray T, Mucke L. Ibuprofen, inflammation and Alzheimer disease. ***Nat Med*** 2000; 6: 973-974.
27. McGeer PL, Schulzer M, McGeer EG. Arthritis and anti-inflammatory agents as possible protective factors for Alzheimer's disease: a review of 17 epidemiologic studies. ***Neurology*** 1996; 47: 425-432.
28. Rogers J, Kirby LC, Hempelman SR, Berry DL, McGeer PL, Kaszniak AW, et al. Clinical trial of indomethacin in Alzheimer's disease. ***Neurology*** 1993; 43: 1609-1611.
29. Minghetti L, Levi G. Microglia as effector cells in brain damage and repair: focus on prostanoids and nitric oxide. ***Prog Neurobiol*** 1998; 54: 99-125.
30. Holmin S, Mathiesen T. Biphasic edema development after experimental brain contusion in rat. ***Neurosci Lett*** 1995; 194: 97-100.
31. Sanders KM. Endogenous prostaglandin E and contractile activity of isolated ileal smooth muscle. ***Am J Physiol*** 1978; 234: E209-E212.
32. Kennedy DW, Abkowitz JL. Mature monocytic cells enter tissues and engraft. ***Proc Natl Acad Sci U S A*** 1998; 95: 14944-14949.
33. Langham J, Goldfrad C, Teasdale G, Shaw D, Rowan K. Calcium channel blocker for acute traumatic brain injury. ***Cochrane Database Syst Rev*** 2003; (3): CD000565.
34. Shen G, Zhou Y, Xu M, Liu B, Xu Y. Effects of nimodipine on changes of endothelin after head injury in rabbits. ***Chin J Traumatol*** 2001; 4: 172-174.
35. Xiao X, Guo X, Wang D, Xue G. Mechanism and treatment principle for cerebral vessel spasm caused by concussion. ***Chin J Traumatol*** 2002; 5: 380-384.
36. Puppo C, Lopez L, Farina G, Caragna E, Moraes L, Iturralde A, et al. Indomethacin and cerebral autoregulation in severe head injured patients: a transcranial Doppler study. ***Acta Neurochir (Wien)*** 2007; 149: 139-149.

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