Supplementary Data

Measures of Lipid Oxidation in Subarachnoid Hemorrhage and Traumatic Brain Injury

Aneurysmal subarachnoid hemorrhage (aSAH) and traumatic brain injury (TBI) patients and their respective controls were comparable (Table 1). However, aSAH patients were significantly older and had a greater prevalence of pre-existing hypertension and hypercholesterolemia than TBI patients ($p < 0.05$). At admission, 12 of the 18 aSAH patients and all 18 of the TBI patients had a Glasgow Coma Score less than 8, indicating severe brain injury. Figure 2 shows isofurans (IsoFs), F_4-neuroprostanes (F_4-NeuroPs) and F_2-isoprostanes (F_2-IsoP) in the cerebrospinal fluid (CSF) of aSAH patients and their respective controls. IsoFs and F_4-NeuroPs were significantly increased in aSAH patients compared with controls and remained significant after adjusting for age and gender ($p < 0.001$ and $p < 0.001$, respectively). Age- and gender-adjusted F_2-IsoP in CSF of aSAH patients were increased compared with controls ($p = 0.011$).

Figure 3 shows CSF IsoFs, F_4-NeuroPs, and F_2-IsoP in TBI patients and their respective controls. IsoFs and F_4-NeuroPs in TBI patients were significantly increased compared with controls and remained significant after adjusting for age and gender ($p < 0.001$ and $p = 0.013$, respectively). CSF F_2-IsoPs were not significantly different between the groups ($p = 0.119$).

There were no significant differences between the aSAH and TBI patients in CSF IsoFs, F_2-IsoPs, or F_4-NeuroPs, after adjusting for age and gender.

Do IsoFs, F_4-NeuroPs, and F_2-IsoP Have a Role in SAH and TBI?

This is the first study to comprehensively examine IsoFs, F_4-NeuroPs, and F_2-IsoPs in the CSF of patients with two catastrophic CNS injuries, namely, aSAH and TBI. In two case-controlled studies, we have shown a significant increase in IsoFs in aSAH and TBI patients compared with their respective age- and gender-matched controls. aSAH patients also had significantly increased levels of F_4-NeuroPs and F_2-IsoPs. Patients with TBI had significantly increased F_4-NeuroPs, but F_2-IsoPs were not different from their controls. These data show that CNS injury as a result of aSAH or TBI results in increased oxidative stress.

Increased concentrations of IsoFs in CSF after an aSAH or TBI are a new and important finding that may have clinical implications. IsoFs are influenced by oxygen tension (5, 6, 14) and altering oxygen concentration is an easily modifiable core component of clinical care in these patients. In contrast to F_2-IsoPs, production of IsoFs continues to rise with increasing ambient oxygen concentrations (5, 14). Previous studies have shown that increased CSF F_2-IsoPs (8) and F_4-NeuroPs (7) correlated with poor neurological outcome in aSAH patients. Whether increased IsoF levels associate with adverse outcomes and whether their concentration can be varied by altering inspired oxygen concentration remains to be determined.

We have shown for the first time that F_4-NeuroPs are significantly increased in the CSF of patients with TBI. Our data also confirm a previous report that F_4-NeuroPs are significantly elevated in the CSF of aSAH patients (7). Since DHA is the major polyunsaturated fatty acid in the brain, F_4-NeuroP levels in CSF are likely a more specific indicator of possible neurological dysfunction than F_2-IsoPs. The F_2-IsoPs derive from oxidative damage to both neural and non-neural cells in the brain, including vascular cells and blood cells, since AA is present in all cells. In this regard, Hsieh *et al.* (7) showed that increased F_4-NeuroPs in CSF of patients with aSAH correlated with poor neurological outcome and suggested that F_4-NeuroPs might be more useful than F_2-IsoPs in CSF to predict outcome and interpret the role of hemorrhage in aSAH.

Our study has shown that CSF F_2-IsoPs in aSAH patients were significantly increased. These data accord with previous work showing that CSF F_2-IsoPs obtained within 24 h of surgery are significantly higher in aSAH patients compared with control patients (8). F_2-IsoPs were also significantly higher in aSAH patients who developed delayed onset cerebral vasospasm (1). Asaeda *et al.* (1) showed that F_2-IsoPs increased during the period of peak vasospasm and decreased gradually thereafter. The authors suggested that increased CSF F_2-IsoPs derived from the subarachnoid clot could be one of the causes of delayed cerebral vasospasm after aSAH. Since F_2-IsoPs promote vasoconstriction and platelet aggregation (2, 11, 14), they may be either mediators or markers of increased vasospasm risk. This might also explain their association with poor outcome (8). It remains to be seen whether therapeutic interventions (cerebral angioplasty) can alter the concentrations of these compounds.

Increased CSF F_2-IsoPs have been shown in adults (15, 17) and infants and children (3) after a TBI compared with controls. Varma *et al.* (16) showed that increased CSF F_2-IsoPs in infants and children with TBI correlated with neuron-specific enolase, a marker of neuronal damage. In contrast, we have shown that CSF F_2-IsoPs were not different between TBI and controls. Differences between previous studies and our findings may relate to methodological differences in the measurement of F_2-IsoPs and/or patient characteristics. The measurement of F_2-IsoPs using mass spectrometry as used by us is considered the gold standard, whereas previous studies (3, 17) have used an enzyme-linked immunoassay kit. We have shown poor agreement between F_2-IsoPs measured using an enzyme-linked immunoassay kit and mass spectrometry (13).

Concluding Remarks and Future Directions

There is an urgent need to identify sensitive and specific markers that will enable the assessment of ongoing CNS injury in deeply comatose patients. CSF IsoFs and F_4-NeuroPs are consistently increased after a catastrophic CNS injury and their measurement may enhance the management of unconscious patients in neurological care. The increase in CSF IsoFs and F_4-NeuroPs in aSAH and TBI patients will require further investigation to establish their association with outcomes and whether they can indicate the efficacy of therapeutic interventions. In particular, increased IsoFs in the CSF of patients

are a new and important finding that may have important clinical implications. IsoFs are altered by oxygen tension, which is an easily modifiable during the clinical care of these patients.

Notes

Patients

In two case-controlled studies, 18 comatose patients with severe aSAH and 18 comatose patients with severe TBI were recruited and matched for age and gender with healthy controls. Controls were adults undergoing subarachnoid anesthesia for elective lower limb surgery. The age difference between aSAH and TBI patients necessitated recruitment of two separate control groups matched for age with the patients. The studies were approved by the human ethics committee of Royal Perth Hospital. Informed written consent was obtained from controls and next-of-kin provided consent for the comatose patients.

CSF sampling

CSF samples were collected from patients within 24 h of the injury from an external ventricular drain inserted, to permit monitoring of intracranial pressures and drainage of CSF. Samples of CSF from control patients were obtained from the subarachnoid space using a spinal needle. CSF was collected into EDTA, reduced glutathione, and butylated hydroxytoluene to prevent *ex vivo* oxidation. Samples were centrifuged at 4°C and stored at −80°C until analysis.

Measurement of CSF IsoFs, F_4-NeuroPs, and F_2-IsoPs

IsoFs, F_4-NeuroPs, and F_2-IsoPs were measured using a modification of our previously reported method (9, 10, 13). Briefly, samples were spiked with 5 ng internal standard 15-F_{2t}-IsoP-d_4 (8-iso-$PGF_{2\alpha}$-d_4) purchased from Cayman Chemicals (Ann Arbor, MI), then hydrolyzed with 1 *M* potassium hydroxide in methanol, acidified, and applied to pre-washed Certify II cartridges (Varian, Lake Forrest, CA). After washing with methanol/water (1:1) and hexane/ethyl acetate (75:25), the metabolites were eluted with ethyl acetate/methanol (90:10) and dried under vacuum. Samples were derivatized using pentafluorobenzylbromide and *N,N*-diisopropylethylamine (Sigma Chemicals, St. Louis, MO), dried under nitrogen, and then treated with the silylating agent *N,O-bis*-(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (Pierce Chemicals, Rockford, IL). IsoFs, F_2-IsoPs, and F_4-NeuroPs were quantitated by gas chromatography–mass spectrometry using electron capture negative ionization and selected ion monitoring. Ions monitored were *m/z* 569, 573, 585, and 593 for F_2-IsoP, 15 F_{2t}-IsoP-d_4, IsoFs, and F_4-NeuroPs, respectively. IsoFs (6) and 4(*RS*)-F_{4t}-NeuroP (4, 12) were synthesized in our laboratory as previously described.

Statistical analysis

Analyses used the STATA (version 11) statistical package (STATA Corp, College Station, TX). CSF IsoFs, F_2-IsoPs, and F_4-NeuroPs were transformed using the natural log before analysis. Differences in CSF IsoFs, F_2-IsoPs, and F_4-NeuroPs between aSAH and controls, and TBI and controls, were analyzed using a general linear model (GLM) with bootstrap estimation of the standard error to accommodate the matched clusters. Differences in CSF IsoF, F_2-IsoP, and F_4-NeuroPs between aSAH and TBI patients were examined using GLM adjusting for age and gender. A *p*-value less than 0.05 was regarded as statistically significant. A power analysis indicated that the sample size of the study would have power in excess of 80% to detect an effect size of 0.5.

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