Beneficial Effect of Methylprednisolone on Cardiac Myocytes in a Rat Model of Severe Brain Injury

MUSTAFA EMIR, KANAT OZISIK,[1] KERIM CAGLI, PINAR OZISIK,[2] SERDAR TUNCER,[3] VEDAT BAKUY, ERKAN YILDIRIM,[1] KAMER KILINC[4] and KAMIL GOL

Department of Cardiovascular Surgery, TYIH, Ankara, and [1]Department of Cardiovascular Surgery, Numune Education and Research Hospital, Ankara, [2]Institute of Neurological Sciences and Psychiatry, [4]Department of Biochemistry, Hacettepe University Medical Faculty, Ankara, and [3]Metis Biotechnology Ltd., Ankara, Turkey

EMIR, M., OZISIK, K., CAGLI, K., OZISIK, P., TUNCER, S., BAKUY, V., YILDIRIM, E., KILINC, K. and GOL, K. *Beneficial Effect of Methylprednisolone on Cardiac Myocytes in a Rat Model of Severe Brain Injury.* Tohoku J. Exp. Med., 2005, **207** (2), 119-124 —— Cardiac injury, occurred after traumatic brain injury (TBI), has been recognized for more than a century. Bcl-2 is a key regulatory component of the mitochondrial cell death pathway, and its overexpression is cytoprotective in many cell types. The therapeutic agents, which induce the expression of bcl-2 protein, might provide a new therapy to prevent cardiac myocyte damage following TBI. In this study, we investigated whether methylprednisolone sodium succinate (MPSS) influences the expression of bcl-2 in the heart. Wistar-Albino female rats underwent TBI (300 g/cm) generated by the weight-drop method, and were left untreated ($n = 6$) or treated with either MPSS (30 mg/kg) ($n = 6$) or vehicle (albumin solution) ($n = 6$). The heart was isolated from each animal with TBI. For comparison, the hearts were isolated from sham-operated ($n = 6$) and control rats ($n = 6$). The relative expression of bcl-2 mRNA in the heart was quantitated by real-time polymerase chain reaction. We also assessed lipid peroxidation in the heart tissue by determining the concentration of thiobarbituric acid-reactive substances (TBARs) as an indicator of tissue damage. The bcl-2 expression level was significantly higher in the hearts of MPSS-treated rats compared to that of other TBI groups ($p < 0.0001$). Moreover, TBI increased the lipid peroxidation in the heart, which was significantly reduced by the treatment with MPSS ($p < 0.0001$). These findings provide evidence for the efficacy of MPSS in protection of cardiac myocytes to achieve optimal heart donation after TBI in heart transplantation. ——— methylprednisolone; bcl-2; apoptosis; TBARs; myocytes; head injuries

Studies on potential clinical donors and on experimental animals have suggested that brain injury can have major histopathological and functional effects on the myocardium (Depasquale and Burch 1969). In animal models of increased intracranial pressure, brain injury results in marked

perturbations of hemodynamics and histological evidence of myocardial damage (Evans et al. 1951; Shivalkar et al. 1993). We have shown that traumatic brain injury (TBI) of 140 g/cm, produced by weight-drop technique, causes obvious gradual damage on ultrastructure of the cardiac myocytes (Ozisik et al. 2004). Pathophysiology of cardiac myocyte injury after severe TBI must be well understood, including the apoptotic processes and free-oxygen radical pathway. These findings are very important in building base of support for the efficacy of pharmalogical agents in protection of cardiac myocytes after TBI.

Bcl-2 family proteins are key regulatory components of the mitochondrial cell death pathway. A number of bcl-2 family proteins are expressed in cardiac myocytes, including bcl-2, bcl-xl, bad, bax, and bid. As in other cells, overexpression of bcl-2 is cytoprotective in cardiac myocytes (Kirshenbaum and de Moissac 1997).

The ability of potentially therapeutic agents to induce the expression of bcl-2 protein might provide a new avenue for therapy to prevent cardiac myocyte damage after TBI. Increasing knowledge of the mechanisms of action indicates that treatment with glucocorticoids could have organ-protective effects. Methylprednisolone sodium succinate (MPSS), a synthetic glucocorticoid, is widely used clinically (Yamanoi et al. 2004) and experimentally (Park 1998) as an acute anti-inflammatory treatment. The molecular actions of MPSS indicate that pretreatment with this drug may be cardioprotective (Valen et al. 2000a). However, MPSS as an antioxidant for the modulator effect on the expression of bcl-2 in myocardial tissue has not been investigated.

MATERIAL AND METHODS

The Institutional Animal Care Committee approved protocols used in this study. Wistar-Albino rats weighing (190-230 g) were randomly allocated into the following groups.

Control group ($n = 6$): Tissue samples were obtained immediately after midline sternotomy and no head surgery was performed.

Sham operated group ($n = 6$): Scalp was closed after craniotomy and no trauma was induced.

Eighteen rats underwent TBI of 300 g/cm and divided into the three groups.

Untreated group ($n = 6$): Rats were left untreated after TBI.

MPSS-treated group ($n = 6$): MPSS in a dose of 30-mg/kg was administered intraperitoneally after TBI.

Vehicle-treated group ($n = 6$): Albumin solution (0.4 ml), containing 2.50 mg human serum albumin, was administered intraperitoneally immediately after TBI.

Tissue samples were obtained from rats 24 h after sham-operation or TBI.

Surgical procedure

The surgical procedure was performed according to the study recently done by our research team (Emir et al. 2004). Rats were anesthetized with 10 mg/kg xylasine (Bayer, Istanbul, Turkey) and 60 mg/kg ketamine hydrochloride (Parke Davis, Istanbul, Turkey). They were then pinned in the prone position, and their scalps were shaved and cleaned with 10% polyvinyl-pyrrolidone/iodine. After midline scalp incision, craniectomy, 5 mm in diameter, was performed with a dental drill system on the right parietal bone of all rats under microscopic visualization. The dura mater was kept intact during this procedure. Trauma was induced using the method described by Allen (1911). The force was applied via a stainless steel rod (3 mm diameter, weighing 30 g) that had rounded surface. The injury apparatus was a 10 cm guide tube, which has 4 mm inner diameter and with holes that allowed the air to go out without making additional pressure effect. It was positioned perpendicular to the burr hole. The weight dropped vertically through the tube from a height of 10 cm onto the exposed dura. Then scalp was closed with 3/0 silk sutures. Body temperature continuously monitored during the procedure with a rectal thermometer was maintained at 37°C, using a heating pad and an overhead lamp. Rats were neither intubated nor ventilated between the brain injury and the heart sampling. None of the animals died during the study period, but injured animals which had no treatment were very sick and hypoactive at the end of the 24 hours.

Sample obtaining from the heart

Twenty four hours after sham operation or trauma, rats were re-anesthetized with the a combination of ketamine and xylasine. Then, the rats were killed by decapitation under general anesthesia. Samples for lipid peroxidation and bcl-2 gene expression were obtained from

the left ventricle of heart. Heart samples were collected in randomly numbered containers and given to the blinded observers. After evaluating the numbered tissues, the results were collected in the appropriate group lists.

Isolation of RNA and synthesis of cDNA

Samples were immediately frozen in liquid nitrogen and kept at –80°C. Total RNA of each myocardial tissue was isolated using a high pure RNA tissue kit (Roche Diagnostics, Germany). RNA integrity electrophoretically verified by ethidium bromide staining and by an OD_{260}/OD_{280} nm absorption ratio > 1.95. One μg of total RNA was used for cDNA synthesis using first strand cDNA synthesis kit for RT-PCR (AMV) (Roche Diagnostics, Germany) according to the manufacturer's protocol.

Quantitative real time PCR analysis

Real time quantitative polymerase chain reaction was performed to analyze bcl-2 mRNA expression, as described previously (Yamada et al. 2002), using a Light Cycler instrument (Roche Diagnostics). The cycling parameters were 2 minutes at 95°C for denaturation, 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C for amplification and quantification. β-actin mRNA was quantified to adjust the amount of mRNA in each sample with the β-actin probe and primer set. The upstream and downstream primers were 5´ TCTTTAATGTCACGCACGATT and 5´ TCACCCACACTGTGCCCAT, respectively. The selected TaqMan probe between the primers was fluorescence labeled at the 5´ end with 6-carboxyfluorescein (FAM) as a reporter dye and at the 3´ end with 6-carboxytetramethylrhodamine (TAMRA) as the quencer 5´-FAM-ATCCTGCGTCTGGACCTGGCT-TAMRA (Tibmolbiol, Germany) (Kalinina et al. 1997; Peirson et al. 2003). Every sample was run for β-actin along with the same cycling protocol described above for bcl-2 gene expression.

Lipid peroxidation assay

The level of lipid peroxidation in the heart muscle was determined using the thiobarbituric acid method of Mihara and Uchiyama (1978). Tissues were homogenized in 10 volumes (w/v) of cold phosphate buffer (pH 7.4). The homogenate (0.5 ml) was mixed with 3 ml of 1% H_3PO_4. After the addition of 1 ml of 0.67% thiobarbituric acid, the mixture was heated in boiling water for 45 minutes. The color was extracted into n-butanol, and the absorption at 532 nm was measured. Using tetramethoxypropane as the standard, tissue lipid peroxidation levels were calculated as nmole/g of wet tissue.

Statistical analysis

All data were coded, recorded, and analyzed by using commercially available statistical software packages. All the continuous data are repesented as mean values ± S.D. Parametric data were compared for differences between groups, by ANOVA with Bonferroni corrections for post-hoc analyses.

RESULTS

Relative expression levels of bcl-2 mRNA were measured in the cardiac myocytes of all animals (Fig. 1). Intraperitoneal administration of MPSS caused a significant increase in the bcl-2 expression ($p < 0.0001$) compared with untreated or vehicle-treated rats. In contrast, treatment with vehicle solution did not cause a significant increase in the bcl-2 expression in cardiac myocytes. These results suggest that MPSS has antiapoptotic effect in cardiac myocytes after severe TBI.

The levels of lipid peroxidation were measured in the heart samples of all animals (Fig. 2). There was no statistically significant difference in lipid peroxidation levels between control and sham-operated animals. TBI caused a significant elevation in lipid peroxidation in untreated or vehicle-treated rats ($p < 0.0001$). Treatment with MPSS significantly decreased TBARs ($p < 0.0001$) compared with other TBI animals. The difference between untreated and vehicle-treated animals was not statistically significant.

DISCUSSION

Glucocorticoids given acutely are benefical against inflammation in various experimental models, and they have been employed to reduce the inflammatory effects of extracorporeal circulation during open heart surgery (Teoh et al. 1995). Increasing knowledge of the mechanisms of action indicate that pretreatment with glucocorticoids could have organ-protective effects. Valen et al. (2000b) showed that treatment with MPSS protected left ventricular function against global
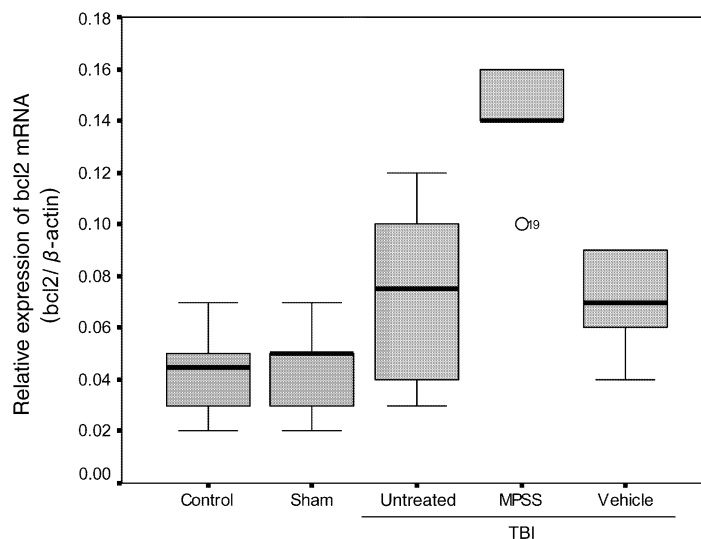
Fig. 1. Effects of TBI on bcl-2 mRNA expression in the heart. The relative expression level of bcl-2 mRNA is shown as ratio of bcl-2/ β-actin. Box extends from Q1 (25%) to Q3 (75%), with the median represented by the horizantal bar within the box. The minimum and maximum values are represented by short horizantal bars to the box by vertical lines. For the MPSS-treated rats, the minimum and maximum values are on the Q1 and Q3, so the short horizantal bars to the box by vertical line are not visible. For this group, an outliner value is shown in the graph. Treatment with MPSS significantly increased bcl-2 expression ($p < 0.0001$) compared with untreated or vehicle-treated animals.
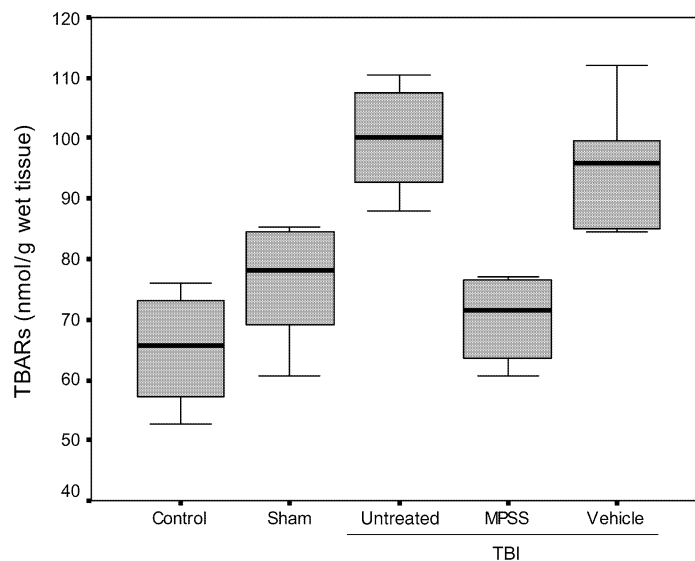

Fig. 2. Effects of TBI on TBARS levels in the heart. Box extends from Q1 (25%) to Q3 (75%), with the median represented by the horizantal bar within the box. The minimum and maximum values are represented by short horizantal bars to the box by vertical lines. Treatment with MPSS significantly decreased TBARS levels ($p < 0.0001$) compared with untreated or vehicle-treated animals.

ischemia and reperfusion in rats.

The process of apoptosis appears to require both an initial extrinsic signal and a complex intrinsic gene regulated apoptotic program. Modulation of the abudance of apoptosis regulators of the bcl-2 family plays a critical role in the determination of cell fate in response to apoptotic stress (Yin et al. 1994). Over expression of bcl-2 has been reported to induce a plethora of apoptosis associated changes, such as alterations of cellular redox state (depletion of glutathione and generation of reactive oxygen species [ROS]), and plasma membrane changes (Minn et al. 1997). We previously demonstrated that erythropoietin may play an important role in the expression of bcl-2 and to decrease TBARs in rat cardiac myocytes after injury of 200 g/cm (moderate TBI) or 300 g/cm (severe TBI). In our previous study, we did not observe any correlation between the degree of bcl-2 expression and the grade of injury (Emir et al. 2004). Therefore, we preferred the severe-grade brain injury induced by weight-drop technique to achieve TBI in rats because of its ability to produce mechanical trauma resembling that occuring in actual life.

Increased bcl-2 levels suggest that the apoptotic cascade after diffuse TBI is a carefully controlled cellular homeostatic response. Pharmalogical manipulation of this balance offer a therapeutic approach for preventing cell death and improving outcome after TBI (Cernak et al. 2002). We found no other papers investigating cardiac myocyte function after intraperitoneal MPSS adminstration. We chose the dose of 30 mg/kg because this treatment protocol was efficient in preserving the function of rat cardiac myocytes, and the because the dose was within clinical range. Our study revealed that the bcl-2 expression ($p < 0.0001$) was significantly higher in MPSS group as compared to other trauma groups, suggesting that MPSS has anti-apoptotic effect in cardiac myocytes after severe TBI.

It is not clear that whether the defence mechanisms of the myocyte are altered or whether the production of free-oxygen radicals is increased, or both. Recent data have shown the immunological response to heart failure results in endothelial and myocyte dysfunction through oxidative stress mediated apoptosis (Ferrari et al. 2004). The lipid peroxidation levels in tissue samples were also evaualted as a secondary indicator of tissue damage. ROS mediated lipid peroxidation is one of the major mechanisms of secondary damage in TBI (Ercan et al. 2001). Hisatomi et al. (1995) showed that the addition of steroid to preservation solution might be effective in preventing myocardial injury during preservation of rat hearts. Radicals can cause damage to cardinal cellular components such as lipids, proteins, and nucleic acids, leading to subsequent cell death by modes of necrosis or apoptosis (Gilgun-Sherki et al. 2002). The increase in TBARs is thought to be a marker of cell damage, which indicates an increased production of ROS and lipid peroxidation. In our study, we found that treatment with MPSS reduced the degree of enhanced levels of TBARs ($p < 0.0001$) in the heart, despite that the follow-up period was only 24 h, suggesting the possible use of MPSS in the protection of myocardial injury after TBI.

The present study provides evidence that MPSS has a preventive effect against apoptosis caused by TBI in rat cardiac myocytes, probably by up-regulating the bcl-2 expression. MPSS also decreases TBARs in the hearts after TBI. These findings underscore the importance of MPSS administration to organ donors and the need for early administration of MPSS to heart donors.

References

Allen, A.R. (1911) Surgery of experimental lesion of spinal cord equivalent to crush injury of fracture dislocation of spinal column. A preliminary report. *JAMA*, **57**, 878-880.

Cernak, I., Chapman, S.M., Hamlin, G.P. & Vink, R. (2002) Temporal characterization of pro- and anti-apoptotic mechanisms following diffuse traumatic brain injury in rats. *J. Clin. Neurosci.*, **9**, 565-572.

Depasquale, N.P. & Burch, G.E. (1969) How normal is the donor heart? *Am. Heart J.*, **77**, 719-720.

Emir, M., Ozisik, K., Cagli, K., Misirlioglu, M., Ozisik, P., Iscan, Z., Yildirim, E., Kilinc, K. & Sener, E. (2004) Effect of Erythropoietin on bcl-2 gene expression in rat cardiac myocytes after traumatic brain injury. *Transplant. Proc.*, **36**, 2935-2938.

Ercan, M., Inci, S., Kilinc, K., Palaoglu, S. & Aypar, U. (2001) Nimodipine attenuates lipid peroxidation during the acute phase of head trauma in rats. *Neurosurg. Rev.*, **24**, 127-130.

Evans, J.P., Espey, F.F., Kristoff, F.V., Kimbell, F.D. & Ryder, H.W. (1951) Experimental and clinical obsevations on ris-

ing intracranial pressure. *Arch. Surg.*, **63**, 107-114.

Ferrari, R., Guardigli, G., Mele, D., Percoco, G.F., Ceconi, C. & Curello, S. (2004) Oxidative stres during myocardial ischaemia and heart failure. *Curr. Pharm. Des.*, **10**, 1699-1711.

Gilgun-Sherki, Y., Rosenbaum, Z., Melamed, E. & Offen, D. (2002) Antioxidant therapy in acute central nervous system injury: current state. *Pharmacol. Rev.*, **54**, 271-284.

Hisatomi, K., Isomura, T., Sato, T., Hayashida, N., Kosuga, K. & Ohishi, K. (1995) Beneficial effect of steroid om myocardial preservation in isolated rat hearts. *Jpn. Circ. J.*, **59**, 815-823.

Kalinina, O., Lebedeva, I., Brown, J. & Silver, J. (1997) Nanoliter scale PCR with TaqMan detection. *Nucleic Acids Res.*, **25**, 1999-2004.

Kirshenbaum, L.A. & de Moissac, D. (1997) The bcl-2 gene product prevents programmed cell death of ventricular myocytes. *Circulation*, **96**, 1580-1585.

Mihara, S. & Uchiyama, M. (1978) Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal. Biochem.*, **86**, 271-278.

Minn, A.J., Velez, P., Schendel, S.L., Liang, H., Muchmore, S.W., Fesik, S.W., Fill, M. & Thompson, C.B. (1997) Bcl-x(L) forms an ion channel in synthetic lipid membranes. *Nature*, **385**, 353-357.

Ozisik, K., Yildirim, E., Kaplan, S., Solaroglu, I., Sargon, M.F. & Kilinc, K. (2004) Ultrastructural changes in of rat cardiac myocytes in a time dependent manner after traumatic brain injury. *Am. J. Transplant.*, **4**, 900-904.

Park, C.O. (1998) The effects of methylpredisolone on prevention of brain edema after experimental moderate diffuse brain injury. *Yonsei Medical Journal*, **39**, 395-403.

Peirson, S.N., Butler, J.N. & Foster, R.G. (2003) Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucleic Acids Res.*, **31**, e73.

Shivalkar, B., Van Loon, J., Wieland, W., Tjandra-Maga, T.B., Borgers, M., Plets, C. & Flameng, W. (1993) Variable effects of explosive or gradual increase of intracranial pressure on myocardial structure and function. *Circulation*, **87**, 230-239.

Teoh, K.H.T., Bradley, C.A., Gauldie, J. & Burrows, H. (1995) Steroid inhibition of cytokine-mediated vasodilatation after warm heart surgery. *Circulation*, **92**, Suppl. II, II347-II353.

Valen, G., Kawakami, T., Tahepold, P., Dumitrescu, A., Lowbeer, C. & Vaage, J. (2000a) Glucocorticoid pretreatment protects cardiac function and induces cardiac heat shock protein 72. *Am. J. Physiol. Heart Circ. Physiol.*, **279**, H836-H843.

Valen, G., Kawakami, T., Tahepold, P., Starkopf, J., Kairane, C., Dumitrescu, A., Lowbeer, C., Zilmer, M. & Vaage, J. (2000b) Pretreatment with methylprednisolone protects the isolated rat heart against ischaemic and oxidative damage. *Free Radic. Res.*, **33**, 31-43.

Yamada, Y., Watanabe, Y., Zhang, J., Haraoka, J. & Ito, H. (2002) Cahnges in cortical and cerebellar bcl-2 mRNA levels in the developing hydrocephalic Rat (LEW-HYR) as measured by a real time quantified RT-PCR. *Neuroscience*, **114**, 165-171.

Yamanoi, T., Shibano, K., Soeda, T., Hoshi, A., Matsuura, Y., Sugiura, Y., Endo, K. & Yamamoto, T. (2004) Intracranial invasive aspergillosis originating in the sphenoid sinus: a successful treatment with high-dose itraconazole in three cases. *Tohoku J. Exp. Med.*, **203**, 133-139.

Yin, X.M., Oltvai, Z.N., Veis-Novack, D.J., Linette, G.P. & Korsmeyer, S.J. (1994) Bcl-2 gene family and the regulation of programmed cell death. Cold Spring Harbor Symp. *Quant. Biol.*, **59**, 387-393.