Abstract. – Background and Objectives: Krabbe disease is a neuro-inflammatory disorder in which galactosyl sphingosine (psychosine) accumulates in nervous tissues. Despite some leads in elucidating the mechanism of psychosine action on different cells of brain, there still remain gaps in the knowledge and mechanisms behind events in the pathogenic cascade of Krabbe disease. Inflammation in the brain in Krabbe disorder is an important factor in neural damage. This study was undertaken to access the role of psychosine in the regulation of nitric oxide (NO) and inducible nitric oxide synthase (iNOS) i.e., inflammatory markers, under two different conditions viz., using a single cell line and using primary mixed glial cells.

Materials and Methods: BV2 murine microglial cells and murine primary mixed glial cells were used during this study. The cell lines, after 12 hr serum starvation, were treated with lipopolysaccharide (LPS) at 25 ng/ml in the absence or presence of increasing concentrations of psychosine (5, 10 and 15 µM). Formation of NO was estimated using Greiss reagent, and expression of iNOS was done by SDS-page followed by western blotting using anti-iNOS antibody.

Results: In BV2 cells it was found that LPS (25 ng/ml) treatment, induced production of NO, but the LPS induced NO production was inhibited when LPS was used in combination with psychosine. This result was corroborated by parallel trend seen in iNOS expression under same conditions. Contrary to this, LPS (25 ng/ml) induced production of NO in primary mixed glial cells was dose dependently enhanced when LPS was used in combination with psychosine (5, 10 and 15 µM). This result was also corroborated by iNOS expression under same experimental conditions.

Discussion: This study suggests that in-vitro data obtained using individual cell lines may not reflect the actual complex intricacies involved in development of Krabbe brain pathology. And that the effect of psychosine in Krabbe brain may be modulated by presence of LPS or other pro-inflammatory stimuli in the brain of these children, e.g., after an infection.
the cells behave same under isolated in-vitro and complex intercellular conditions. We sought to find whether the response shown by BV-2 cells to psychosine induced nitric oxide synthesis to that shown by a mixed glial culture, which more closely represents the in-situ brain conditions.

**Materials and Methods**

**Chemicals**
Lipopolysaccharide (LPS; 0111:B4)) from *Escherichia coli*, sodium nitrite, N-(1-naphthyl) ethylenediamine, sulfanilamide and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma-Aldrich Chemical Corporation (St. Louis, MO, USA). Antibody against iNOS and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Psychosine was purchased from Matreya (Pleasant Gap, PA, USA).

**Animals**
Animal work was performed under a protocol approved by the Institutional Animal Care and Use Committee of the University of Kashmir, Srinagar, J&K, India. C57BL/6J were purchased from IIIM, Jammu (India) and maintained at the University’s animal facility.

**Cell Culture**
BV2 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) (4.5 g glucose/L) supplemented with antibiotics viz., penicillin (20 U/mL), streptomycin (20 mg/mL), and 10% heat-inactivated *Fetal bovine serum* (FBS, GIBCO®, Grand Island, NY, USA). They were then subcultured in 6-well plates, and were serum starved overnight and then treated with LPS (25 ng/ml) or LPS (25 ng/ml) + psychosine (5-15 µM; increasing concentrations), and 24 hr later, supernatant was collected for the determination of NO production. Primary mixed glia cultures were prepared from postnatal day 1-3 (P1-P3) mouse brains (C57BL/6J)\(^{15,16}\).

**Western Blot Analysis**
Western blot analysis for inducible nitric oxide synthase (iNOS) and β-actin expression was performed using standard procedures. Briefly, treated BV-2 cells or primary mixed glia were harvested and lysed with lysis buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 5 mM EDTA, 50 mM NaF, and 0.5% Nonidet P-40) containing a protease inhibitor cocktail (Sigma). The lysate was clarified by centrifugation at 10,000 g for 15 min at 4°C. After determining the concentration of the protein in the samples, equal protein concentration (50 µg) was loaded in each well and subjected to sodium dodecyl sulfate-PAGE on a precast 4-20% gel from Bio-rad (Bio-rad Laboratories Inc., Hercules, CA, USA) for 1hr at 25 mA. After the gel was run, the proteins in the gel were electrophoretically transferred to a high-bond nitrocellulose membrane (Amersham Life Science, Arlington Heights, IL, USA), using gel transfer apparatus (Bio-rad Laboratories Inc., Hercules, CA, USA), for western blotting purpose. After transfer, blocking was performed with Tween 20-Tris-buffered saline (20 mM Tris-HCl, pH7.6,
137 mM NaCl and 0.05% Tween-20) containing 5% non-fat milk for 1h at room temperature, the membranes were incubated overnight at 4°C with the primary antibody at 1:1000 dilution in blotting buffer (Tween-20 Tris-buffered saline with 5% non-fat milk). The membranes were then washed three times for 10 min each in Tween 20-Tris-buffered saline and incubated with an appropriately diluted horseradish peroxidase-labeled secondary antibody (1:2000) in blotting buffer for1h at room temperature. The membranes were washed three times, reacted with enhanced chemiluminescence reagent (Amersham Life Science, Arlington Heights, IL, USA) and subjected to autoradiography. Protein levels were standardized by comparison with β-actin expression under similar conditions.

**Statistical Analysis**

The values are expressed as mean ± SD of n determinations, as mentioned under figures. The results were analyzed statistically by student’s t-test using Graphpad prism 5 software. Results were considered statistically significant at p value of less than 0.05.

**Results**

**Effect of Psychosine on the NO Production and iNOS Expression in BV-2 Cells**

After overnight serum starvation, BV2 cells were treated with LPS (25 ng/ml) or LPS (25 ng/ml) + psychosine (5-15 µM; increasing concentrations), and 24 hr later, supernatant was collected for the determination of NO production. The Cells were lysed for the iNOS expression by western blotting. It was seen that the LPS induced NO production decreased with the psychosine treatment in a concentration dependent manner (Figure 1A). This result was also supported by the decrease in LPS induced iNOS expression by increasing concentrations of psychosine (Figure 1B).

**Effect of Psychosine on the NO Production and iNOS Expression in Primary Mixed Glial Cells**

After overnight serum starvation, cells were treated with LPS (25 ng/ml) or LPS (25 ng/ml) + psychosine (5-15 µM; increasing concentrations), and 24 hr later, supernatant was collected for the determination of NO production. The cells were lysed for the iNOS expression by western blotting. It was seen that LPS at the concentration of 25 ng/ml was not able to induce the NO production in the primary mixed glial cells, and that the psychosine dose dependently increased the NO production (Figure 2A). This result was further corroborated by the finding that the iNOS expression by LPS was undetectable, and that the psychosine dose dependently increased its expression (Figure 2B).

**Discussion**

In globoid cell leukodystrophy (GLD), also called Krabbe’s disease, the primary defect is an absence of the lipid-degrading enzyme galactocerebromidase, which cleaves the galactose headgroup from galactoceramide (Figure 3). Unlike some other lipidoses, the primary substrate of the missing enzyme, galactoceramide, does not accumulate. Instead, a related lipid metabolite, psychosine (Figure 3), accumulates in the brain18. Suzuki7 hypothesized that psychosine is normally broken down by galactoceramidase, and that in its absence psychosine accumulates, causing death of oligodendrocytes. These are the cells that normally synthesize galactoceramide during myelination, so their death would account for the absence of galactoceramide buildup in GLD pathology. This “psychosine hypothesis” has stood the test of time, the multinucleate globoid cells in GLD are thought to derive from microglia and macrophages19. The exact mechanism by which psychosine acts remains a mystery till date, whether psychosine action leads to the induction of inflammatory markers or whether the inflammatory markers augment the action of psychosine is still to be resolved.

The present study although a minor step was aimed to find whether the data collected on individual cell lines can be the substitute to the probable action exerted by psychosine in a multicellular set up. Since it is difficult to access the action of psychosine per-se on the brain cells in-vivo, we have tried to investigate the psychosine action on murine macrophage cell line versus its action on murine primary mixed glial cells in-vitro.

It has been widely reported that LPS induces the NO production and iNOS expression in a large variety of cell lines20-22, particularly the macrophage cell lines, and the iNOS induction in brain is also thought to be primarily contributed by microglia13,24. Reports describing the relative contributions of microglia vs. astrocytes to the production of NO differ13,25,26. The induction
**Figure 1.** Nitric oxide (NO) and inducible nitric oxide synthase (iNOS) expression in BV2 cells. After overnight serum starvation, BV2 cells were treated with LPS (25 ng/ml) in the absence or presence of increasing concentrations of psychosine (5-15 µM), after 24 hr, the production of nitric oxide was estimated in culture supernatants (1A), and western blot analysis for iNOS expression was done in cell lysates (1B). Abbreviations used: LPS; lipopolysaccharide, P5, P10 and P15 represent 5, 10 and 15 µM of psychosine, control represents vehicle i.e., dimethylsulfoxide (DMSO) only. Values are mean + SD of 3 independent observations, each done in triplicate. **P < 0.01 and ***P < 0.001 as compared to control.

**Figure 2.** Nitric oxide (NO) and inducible nitric oxide synthase (iNOS) expression in murine primary mixed glial (PMG) cells. After overnight serum starvation, PMG cells were treated with LPS (25 ng/ml) in the absence or presence of increasing concentrations of psychosine (5-15 µM), after 24 hr, the production of nitric oxide was estimated in culture supernatants (2A), and western blot analysis for iNOS expression was done in cell lysates (2B). Abbreviations used: LPS; lipopolysaccharide, P5, P10 and P15 represent 5, 10 and 15 µM of psychosine, control represents vehicle i.e., dimethylsulfoxide (DMSO) only. Values are mean + SD of 3 independent observations, each done in triplicate. **P < 0.01 and ***P < 0.001 as compared to control.
of iNOS expression in astrocytes has been reported. However, the level of NO in interferon-γ/LPS-stimulated purified astrocyte cultures has been reported to be unmeasurable. The present study was carefully designed using a low concentration of LPS to make the psychosine effect prominent. We found that both NO production and iNOS induction by psychosine in microglia BV2 cell line was exactly opposite to its effect in primary mixed glial cells. While as in BV2 cell line psychosine decreased the LPS induced iNOS production, in mixed glial cell culture LPS alone did not induce iNOS but led to its induction in a dose dependent manner by psychosine.

Due to variable forms of onset of clinical signs in patients with Krabbe disease and the lack of correlation between disease progression and enzyme levels or mutation status, other factors, such as environmental and/or other genetic factors, have been suggested to influence the late onset of Krabbe disease. Environmental factors include the possibility that trauma, i.e., a blow to the head, or viral infections may trigger the onset of symptoms in humans with Krabbe disease, perhaps by induction of proinflammatory mediators. It is pertinent to mention that people have reported the accumulation in inflammatory cytokines like TNF-α and IL-6 in Krabbe mice model and human brain samples from GLD patients.

We propose that since BV2 cells are microglial in nature the inhibitory action of psychosine on NO and iNOS may be explained in part due to the functional-inactivation of these cells by conversion into foam cells, this action of psychosine on microglial cells is well documented. However, when using a mixture of astrocytes, oligodendrocytes, microglia as represented by primary mixed glial culture, the effect of psychosine is different in part as the cells in concert modulate the function of each other. Our results of non-induction of NO and iNOS in mixed glia is in line with the already documented reports about iNOS inhibition in microglial by astrocytes in mixed cultures.

In conclusion, one may have to remain cautious while extrapolating the in-vitro results from individual cell lines to that of mixed primary cell cultures; the latter may more closely mimic the cellular milieu inside the brain. Therefore, the present investigation makes a small but important contribution in recognizing that the individual effects of psychosine in-vitro on individual cell lines may not exactly represent its action during its accumulation in vivo in a complicated disease like Krabbe.

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References

Differential iNOS regulation by psychosine


