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Influx and Efflux of Glutathione During Continuous Pain Induction in Rat Hepatocytes and Glial Cells

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Abstract: In this study we examined the intra and extracellular glutathione levels in both glial cells and hepatocytes of rats during continuous pain induction. Animals were divided in 4 groups; one day, four day and seven day pain groups vs. control group. Results showed that the maximum generation of ROS occurs in four day group of continuous pain induction in both glia and hepatocytes of rats. The intracellular GSH was at highest level in one day pain group due to GSH influx and synthesis as two defensive mechanisms against oxidative stress in both glia and hepatocytes. In hepatocytes, however, by considering the levels of extracellular GSSG in one day pain group, it seems that the enzymatic function of GSSG reductase is also another important defense mechanism in the first group of continuous pain induction. Maximum extra cellular GSSG was in 7 day pain group in both glia and hepatocytes. Results suggest that following continuous pain induction, oxidative stress is the major cause for GSH depletion in both glia and hepatocytes and leads cells to the later consequences including apoptosis.

Key words: Inflammatory pain, glutathione, reactive oxygen species, apoptosis, rat

INTRODUCTION

Subcutaneous (s.c.) injection of dilute formalin into a hind paw induces a rapid and prolonged hyperalgesia across widespread areas of the body. This s.c. chemical irritant activates peripheral nerves leading to activation of dorsal horn neuron[1-3].

Glial cells (astrocytes and microglia) in the brain and spinal cord have been recognized as active participants in the creation and maintenance of continuous pain induced by inflammation, damage to peripheral tissues, peripheral nerves, spinal nerves and the spinal cord. On activation, this glia express characteristics quite similar to immune cells, in which they respond to viruses and bacteria, releasing pro-inflammatory cytokines which create pathological pain[4,5].

This CNS glia also becomes activated by certain sensory signals arriving from the periphery, leading to release of pro-inflammatory cytokines. To date, most evidence has supported a putative role for the glial pro-inflammatory cytokines, tumor necrosis factor (TNF-α), interlukin-1 (IL-1) and IL-6[6,7].

Proinflammatory cytokines are classically known as a family of proteins released by activated immune cells. It is an important feature that the effects of proinflammatory cytokines will be synergized (especially TNF-α and IL-1) if released together, that more powerful effects are observed when more than one cytokine is present. TNF-α and IL-1 in particular are very potent biological molecules, producing large effects when administered in the CNS in the femptogram to picogram range[6,8,9].

Following activation, glia cause pain transmission neuron hyperexcitability and the exaggerated release of substance P and EAAs (excitatory amino acids) from presynaptic terminals. These changes are created by the glial release of NO, EAAs, ROS (Reactive Oxygen Species), PGs (prostaglandins), pro-inflammatory cytokines (for example IL-1, IL-6 and TNF-α) and NGF (nerve growth factor)[7-9].

This massive release of proinflammatory cytokines could induce some levels of damage to the neighboring neurons and glial cells in the brain and spinal cord. There is therefore more to investigate the molecular and pathological mechanisms involved in the continuous pain induced brain, spinal cord or liver injury.

Hepatocytes express and release inflammatory mediators after challenge with proinflammatory cytokines released by the glial cells during the inflammatory pain. Nitric oxide synthase-2 (NOS-2) is expressed under these conditions and the high-output

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NO synthesis that follows, contributes to the inflammatory response in this tissue and can be a cause for several hepatopathies[10].

Glutathione (GSH) is a ubiquitous essential tripeptide that protects cells against oxidants, electrophilic compounds and xenobiotics. It is a key intracellular reducing agent and is implicated in immune modulation and inflammatory conditions.

Proinflammatory cytokines especially TNF-α induce oxidative stress by the generation of ROS via leakage from the mitochondria electron transport chain associated with an increase in a variety of defense mechanisms including antioxidants. These inflammatory events lead to GSH depletion and increasing of oxidized GSH (GSSG)[8,11,12].

The purpose of this study was to examine the reduced and oxidized glutathione levels of glial cells and hepatocytes during continuous inflammatory pain in rats, as a marker for determination of pain induced brain or liver damage.

MATERIALS AND METHODS

Animals: Male wistar rats (200-300 g) were used in the present study. All rats were housed in a room at a constant temperature of 25°C on a 12/12 h light/dark cycle with food and water available *ad libitum*. All experiments were conducted according to protocols approved by the Committee of Animal Experimentation of Shaheed Beheshti University of Medical Sciences, Tehran, Iran. This study was performed in Faculty of Pharmacy, Shaheed Beheshti University of Medical Sciences from September 2003 to September 2004.

Pain induction and grouping: A subcutaneous (s.c) injection of 50 μL of 5% formalin in to one hind paw was used for induction of continuous pain[1,3].

Animals were grouped as:

- Animals suffered inflammatory pain for 1 day. This group received a single injection in to a one side of hind paw.
- Animals suffered inflammatory pain for 4 days. For this group, the procedure mentioned for group one was repeated for 4 consecutive days and every day, the formalin injection was given in to a different paw site (day 1: right/dorsal, day 2: left/ventral, day 3: right/ventral, day 4: left/dorsal).
- Animals suffered inflammatory pain for 7 days. In this group, the same procedure was carried out 4 times on each side of hind paws but a day interval was considered for each injection.

Cell preparation

Freshly prepared hepatocytes:
Hepatocytes were isolated from adult male wistar rats by collagenase perfusion of the liver as described by Pourahmad and O'Brien[13]. Cell viability was measured by Trypan blue exclusion method and the viability considered in this study was at least 85-90%.

Glial cells: Glial cells were prepared from hippocampus of wistar rats adapted from Dermietzel *et al.*[14]. In brief after removal of hippocampus it was collected in Phosphate Buffered Saline (PBS) with pH 7 and afterward transferred to trypsin-EDTA(0.1%) and dissected to small parts, incubated in 37°C for 10 min.

After cell dissociation, DMEM medium was added and passed through a 70 and 25 μm nylon mesh.

GSH and GSSG assessment: GSH and GSSG were determined according to the spectrofluorimetric method[15]. Each sample was measured in quartz cuvettes using a fluorimeter set at 350 nm excitation and 420 nm emission.

Determination of ROS: To determine the amount of ROS generation, 2',7'-dichlorofluorescin diacetate was used as it penetrates the cells and becomes hydrolyzed by an intracellular esterase to form DCFH. The latter reacts with intracellular ROS to form the highly fluorescent 2',7'-dichlorofluorescin, which effluxes the cell. The fluorescence intensity of the 2',7'-dichlorofluorescin formed was determined at 540 nm (emission) and at 470 nm (excitation)[16].

Statistical analysis: Data were analysed using one-way analysis of variance (ANOVA) and Levene´s test for homogeneity of variances. Results represent the mean±SD of triplicate samples. The minimal level of significance chosen was $p=0.001$[17].

RESULTS

As shown in Table 1 and 2, pain stress significantly ($p=0.001$) depleted hepatocytes and glial cells intracellular GSH in 4 and 7 day pain groups comparing to the corresponding control groups. In 1 day pain group, GSH level however was significantly raised in both hepatocytes and glia. Pain stress lowered the glial cells extracellular GSH levels in all groups. On the other hand, in hepatocytes, extracellular GSH levels only showed significant increase ($p=0.001$) in 1 day pain group comparing to control, 4 and 7 day pain groups (Table 1).

Table 1: Intra and extracellular levels of reduced and oxidized glutathione of hepatocytes in different pain groups of rats

	Reduced glutathione (GSH) (μM)		Oxidized glutathione (GSSG) (μM)	
Groups	Intracellular	Extracellular	Intracellular	Extracellular
Control	18.0±2	22.0±2	8.0±1	170.0±8
1 day	65.0±5[a]	37.0±3[a]	41.0±4[a]	53.0±5[a]
4 day	50.0±5[ab]	23.0±3[b]	180.0±12[ab]	312.0±15[ab]
7 day	10.0±1[abc]	22.0±2[b]	121.0±8[abc]	432.0±15[abc]

Values are expressed as mean±SD and analyzed using ANOVA followed by Tukey test. Hepatocytes (10^6 cells mL^{-1}) were incubated in Krebs-Henseleit buffer pH 7.4 at 37°C (13).
[a]Significant difference in comparison with control hepatocytes (p=0.001)
[b]Significant difference in comparison with one day pain group (p=0.001)
[c]Significant difference in comparison with four day pain group (p=0.001)

Table 2: Intra and extracellular levels of reduced and oxidized glutathione of glia in different pain groups of rats

	Reduced glutathione (GSH)(μM)		Oxidized glutathione (GSSG)(μM)	
Groups	Intracellular	Extracellular	Intracellular	Extracellular
Control	69.0±5	324.0±15	5.0±1	177.0±12
1 day	107.0±7[a]	234.0±12[a]	45.5±5[a]	265.0±11[a]
4 day	22.0±4[ab]	79.0±5[ab]	212.0±9[ab]	303.0±15[a]
7 day	23.0±3[ab]	72.0±8[ab]	223.0±9[ab]	402.0±15[abc]

Values are expressed as mean ± SD and analyzed using ANOVA followed by Tukey test. Glial cells (10^6 cells mL^{-1}) were prepared by dissection of hippocampus, dissociation of cells in trypsin-EDTA at 37°C and passing through a 70 and 25 μm nylon mesh in DMEM medium(14).
[a]Significant difference in comparison with control hepatocytes (p=0.001)
[b]Significant difference in comparison with one day pain group (p=0.001)
[c]Significant difference in comparison with four day pain group (p=0.001)

GSSG levels were significantly (p=0.001) increased in both cell types following the pain induction. Only in the 7 day pain group, the hepatocytes intracellular GSSG levels were significantly lower than that of the 4 day pain group (Table 1 and 2).

The extracellular GSSG levels in both glia and hepatocytes in 4 and 7 day pain groups were significantly (p=0.001) higher than those of corresponding control groups (Table 1 and 2). Although the extracellular GSSG levels in glial cells of 1 day pain group were significantly (p=0.001) higher than that of control group but there was a significant decrease in the extracellular GSSG levels in hepatocytes of 1 day pain group comparing to its control (Table 1 and 2).The order of extracellular GSSG increase in different groups was as follows:

In glial cells: 7 day group >4 day group >1 day group > control

In hepatocytes: 7 day group >4 day group > control >1 day group

Figure 1 shows the comparative levels of ROS formation in glia and hepatocytes of control and three different pain groups. The increase in ROS generation was demonstrated as fold(s) to ROS levels by the corresponding control group. There was a significant increase in ROS formation of both glia and hepatocytes

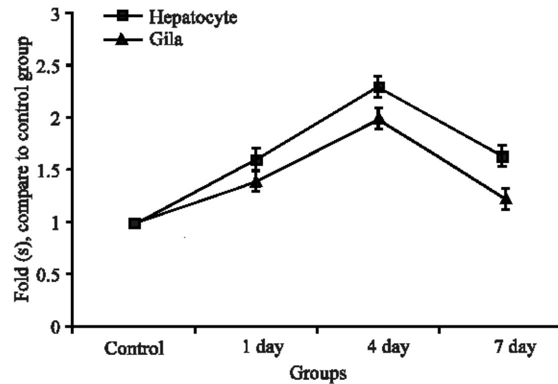

Fig. 1: Demonstrates comparative levels of Reactive Oxygen Species formation in glia and hepatocytesgroups. All groups were significantly different (p=0.001) from each other except for (1 vs. 7 day groups) in both cell types; ANOVA followed by Tukey test

following the pain induction in 1 and 4 day pain groups. However, ROS formation was significantly lower in 7 day pain group comparing to 4 day pain group.

DISCUSSION

The GSH redox system is crucial in maintaining intracellular GSH homeostasis, which is critical to normal cellular physiological process and represents one of the most important antioxidant defense systems in the body. This system uses GSH as a substrate in the detoxification of peroxides such as hydrogen peroxide (H_2O_2) and lipid peroxides, a reaction that involves glutathione peroxidase (GPx). This reaction generates oxidized GSH (GSSG), which is subsequently reduced by GSSG reductase in a reaction that requires the hexose monophosphate shunt pathway producing NADPH. Physiologically the GSSG reductase reaction is driven strongly in favor of GSH, with the GSH to GSSG ratio normally more than 90%[8,18].

In the present study it is investigated that the inflammatory pain induced intracellular GSH alterations and ROS generation in the rat brain glial cells and hepatocytes. Results showed the intracellular GSH levels were significantly increased in both glial cells and hepatocytes in the one day pain group (Table 1 and 2). On the other hand the intracellular ROS formation was also significantly increased in the both hepatocytes and glial cells of all rats of the one day pain group comparing to control group (Fig. 1), it suggest that oxidative stress or in other word, ROS formation is rapidly started following the inflammatory pain induction in both brain and liver cells. It is also suggested from results that the intracellular defense mechanism including GSH synthesis[19] and GSH

influx[20,21] and also GSSG reduction to GSH by GSSG reductase[8], are reflectively activated following the pain induction.

Since the extracellular GSSG contents were significantly decreased in all rats of one day pain group we therefore suggest that the enzymatic function of GSSG reductase is the major defense mechanism in the first day following the inflammatory pain induction in the hepatocytes. Then the reduced GSH could influx the cell to cope with ROS formation and oxidative stress. On the other hand, the increase in GSH synthesis and also influx, are the major defense mechanisms in the brain glial cells following the inflammatory pain induction (Table 1 and 2). Two cytosolic enzymes, γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase (GS), are involved in GSH synthesis. γ-GCS catalyses the rate limiting state in the GSH synthesis[18]. In the physiologic conditions, about the 80% of γ-GCS is inactive due to binding to GSH. In the oxidative stress condition, the enzyme is released from GSH bound and then be activated[8,18]. The GSH synthesis will therefore be considerably increased. This defensive process is one of the ways that a normal cell copes with the oxidative stress and compensates its depleted GSH. Besides it is now clear that under the oxidative stress condition the γ-GCS mRNA will be upregulated. It has already been reported that the intracellular GSH, was significantly increased in 24 h following the oxidative stress induction on the lung alveolar epithelial cells[18,22]. This result is similar to the results of one day pain group. Results also showed (Table 1 and 2) in all rats of 1 day pain group that the intracellular GSSG contents were significantly increased and then transferred to extracellular fluid using the known membrane channels[23], suggesting that the ROS formation induced was responsible for GSH oxidation.

Maximum intracellular ROS formation in this study was reported in both rat hepatocytes and glial cells of 4 day pain group (Fig. 1), suggesting that pick of oxidative stress following the continuous pain induction could be occurred in the 4th day. Obviously activation of different defense mechanisms could then justify decrease in intracellular ROS formation in both rat hepatocytes and glial cells of 7 day pain group.

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