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Original Article

Impact of Duration and Severity of Persistent Pain on Programmed Cell Death

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Abstract

Programmed cell death is a highly regulated form of cell death, mostly distinguished by the activation of a family of cystein-aspartate proteases (caspases) that cleave various proteins resulting in morphological and biochemical changes characteristic of this form of cell death. Several recent studies have addressed the role of programmed cell death in inflammatory and chronic pain states. Caspase-3 plays a central role in mediating nuclear programmed cell death including chromatin condensation and DNA fragmentation as well as cell blebbing. The aims of this study were to investigate the effect of duration and severity of persistent pain on the induction of programmed cell death. Formalin was administered subcutaneously in the Wistar rat hind paws for 1, 4 or 7 consecutive days, and then the activity of caspase-3 was measured in both the rat liver and brain cells. Morphological changes characterizing programmed cell death were also studied using the Sigma's Apoptosis Detection kit, Annexin V-Cy3. Our findings showed that caspase-3 activity and apoptotic phenotype significantly increased in liver but not brain cells following the increase in duration and severity of formalin induced persistent pain.

Keywords: Inflammatory pain; Reactive oxygen species; Caspase, programmed cell death; Glia; Hepatocytes; Rat.

Introduction

The glia become activated by certain sensory signals arriving from the periphery, leading to the release of pro-inflammatory cytokines. One of them is the signals arising from the subcutaneous injection of dilute formalin into hind paw (1, 2).

The triggers for initiation of the responses are substances released by immune cells activated by a foreign entity. As a group, these proteins are referred to as pro-inflammatory cytokines (2). Following activation, glia causes pain transmission neuron hyperexcitability, the exaggerated release of substance P and excitatory amino acids (EAAs) from presynaptic terminals. These changes are created by the glia release of NO, EAAs, reactive oxygen species (ROS), prostaglandins, pro-inflammatory cytokines (especially IL-1, IL-6 and TNF-α) and nerve growth factor (1).

This massive release of pro-inflammatory cytokines could induce some levels of damage to the neighboring neurons and glial cells in the brain and spinal cord. Other organs such as liver that express and release inflammatory mediators after challenge with pro-inflammatory cytokines released by the glial cells during the inflammatory pain can be damaged, specially when the largest mass of macrophages in the

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body (kupffer cells) are present in the liver and could have two opposing roles: a source of inflammatory mediators and the target organ for the effects of these inflammatory mediators (3, 4).

Cellular sources of ROS production include plasma membrane NADPH oxidase and intracellular cytosolic xanthine oxidase, peroxisomal oxidases, endoplasmic reticular oxidases, mitochondrial electron transport components and lysosomal pool of Fe^{++}/Cu^{+} which makes it susceptible for Haber-Weiss reaction with H_2O_2 generating agents (5, 6). The two latter agents are considered to be the major sources of ROS that have been implicated in a number of diseases and disorders (6, 7).

It is clear that cytokines can induce oxidative stress by the generation of ROS via leakage from the mitochondrial electron transport chain (3). The major consequence of ROS formation and oxidative stress is triggering the processes that lead to programmed cell death (apoptosis) (3, 8). Much of the available publications suggest that the ROS involved in apoptosis signaling are the consequence of an impairment in the mitochondrial respiratory chain (9, 10).

It is clear that caspases are fundamental to apoptotic regulation. These are cysteine proteases that are believed to serve as effector molecules of apoptosis, operating through proteolytic activation to precipitate the death response. Caspases are constitutively expressed as inactive procaspases found in the cytosol, and are activated by proteolytic cleavage of inhibitory sequences in response to apoptotic signals (11, 12).

The aims of this study were to evaluate ROS formation, glutathione depletion and caspases activity in asterocytes and hepatocytes following formalin induced inflammatory pain in rats.

Experimental

Animals

Male wistar rats (200-300 g) were used in the present study. All test and control groups contained ten rats. 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 the 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 into one hind paw was used for the induction of continuous pain (13,14).

Animals were grouped as:

a) Animals suffered inflammatory pain for 1 day. This group received a single injection into one side of the hind paw.

b) 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 into a different paw site (day1: right/dorsal, day2: left/ventral, day3: right/ventral, and day 4: left/dorsal).

c) Animals suffered inflammatory pain for 7 days. In this group, the same procedure was carried out 4 times on each side of the hind paws but a day interval was considered for each injection.

d) Control group. In this group, animals suffered no pain.

Cell preparation

Freshly prepared hepatocytes:

Hepatocytes were isolated from adult male wistar rats by collagenase perfusion of the liver as decribed by Pourahmad and O'Brien (15). Cell viability was measured by the 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.* (16). In brief, after removal of the 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 at 37°C for 10 min. After cell dissociation, DMEM medium was added and

passed through a 70 μm and then a 25 μm nylon mesh.

GSH and GSSG assessment

GSH and GSSG were determined according to the spectrofluorimetric method (17). 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 2′,7′-dichlorofluorescin. The latter reacts with intracellular ROS to form the highly fluorescent 2′,7′-dichlorofluorescein, which effluxes the cell. The fluorescence intensity of the 2′,7′-dichlorofluorescein formed was determined at 470 nm (emission) and 540 nm (excitation) (18).

Determination of caspase 3 activity

Caspase 3 activity was determined in cell lysate of hepatocyte and glial cells from different groups, using the ″Sigma's caspase 3 assay kit (CASP-3-C)″ (Sigma-Aldrich, Taufkirchen, Germany). In brief, this colorimetric assay is based on the hydrolysis of substrate peptide by caspase 3. The released moiety (p-nitroaniline) has a high absorbance at 405 nm. The concentration of the p-nitroaniline released from the substrate is calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined p-nitroaniline solutions.

Detection of apoptosis

Apoptosis was the detected using the ″Apoptosis Detection Kit, Annexin V-CY3″ purchased from Sigma. Briefly, in this kit two labels were used: 6-carboxyfluorescein (6-CF) was observed as green and Annexin V-Cy3 (AnnCy3) as red fluorescence. After labeling at room temperature, the cells were observed by fluorescence microscopy. Live cells were stained only with 6-CF (green), while necrotic cells stained only with AnnCy3 (red). Cells starting the apoptotic process were stained with both AnnCy3 and 6-CF.

Statistical Analysis

Levene´s test was used for homogeneity of variances. Data were analysed using one-way analysis of variance (ANOVA) followed by Tukey post-test. Results represent the mean ± standard deviation of the mean (S.D) of triplicate samples. The minimal level of significance chosen was $P \leq 0.05$ (19).

Results and discussion

As shown in figure 1, continuous pain significantly increases the levels of ROS formation in glia and hepatocytes of control and three different pain groups. ROS level was shown as fold(s) to the levels by the corresponding control group. In both glia and hepatocytes, a significant increase in ROS formation following the pain induction in 1 and 4 day pain groups is noticed. It suggests that oxidative stress (ROS formation) is rapidly started following the inflammatory pain induction in both glial cells and hepatocytes. In certain levels, ROS are necessary for metabolism of aerobic organisms, but beyond this, they will cause oxidative stress (20, 21).

Obviously, activation of different cellular defense or resistance mechanisms could then justify a decrease in intracellular ROS formation in both rat hepatocytes and glial cells of 7 day pain group (22).

Glutathione (GSH) is a ubiquitous thiol-

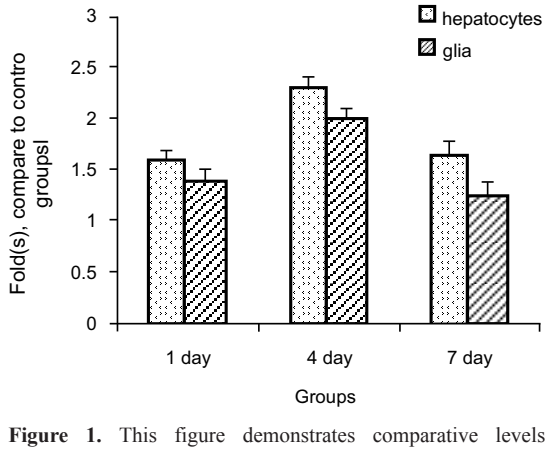

Figure 1. This figure demonstrates comparative levels of Reactive Oxygen Species (ROS) formation in glia and hepatocyte groups. All groups were significantly different ($P \leq 0.001$) from each other except for 1 vs. 7 days groups) in both cell types based on the; ANOVA followed by Tukey test.

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

Groups	Reduced glutathione (GSH) (μM)		Oxidized glutathione (GSSG)(μM)	
	Intracellular	Extracellular	Intracellular	Extracellular
Control	0.14 ± 0.01	0.16 ± 0.01	0.089 ± 0.01	2 ± 0.09
1 day pain induction	0.49 ± 0.04[a]	0.28 ± 0.02[a]	0.48 ± 0.05[a]	0.62 ± 0.06[a]
4 day pain induction	0.37 ± 0.04[a,b]	0.17 ± 0.02[b]	2.12 ± 0.14[a,b]	3.68 ± 0.18[a,b]
7 day pain induction	0.07 ±0.01[a,b,c]	0.16 ± 0.01[b]	1.43 ± 0.09[a,b,c]	5.09 ± 0.18[a,b,c]

Values are expressed as mean ± SD and analyzed using ANOVA followed by Tukey test.
Hepatocytes (106 cells/ml) were incubated in Krebs-Henseleit buffer pH 7.4 at 37° C (13).
Significant difference in comparison with a: control hepatocytes (P≤ 0.001); b: one day pain group (P≤ 0.001); c: four days paingroup (P≤ 0.001), (n=10).

containing tripeptide, which plays a key role in cellular defense against xenobiotics and naturally occurring deleterious compounds such as free radicals and hydroperoxides. GSH levels are a highly sensitive indicator of cell functionality and viability (23). The overwhelming level of intracellular ROS and GSSG (oxidized form of GSH) indicates a disturbance in the Redox status of the cell, a condition that may be followed by apoptosis (23).

Tables 1 and 2 show the intr- and extracellular levels of glutathione (GSH) in different pain induced groups of glia and hepatocytes. As shown, pain stress significantly depleted intracellular GSH in 4 and 7 days pain groups comparing to the corresponding control group in both hepatocytes and glia. 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. However, in hepatocytes, extracellular GSH levels only showed a significant increase in the 1 day pain group comparing to the control, 4 and 7 days pain groups. The order of extracellular oxidized glutathione (GSSG) increase in different groups was as follows:

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

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

Our results suggest that the intracellular defense mechanisms including GSH synthesis, GSH influx and GSSG reduction to GSH by GSSG reductase, are reflectively activated following the pain induction (22).

The execution of apoptosis appears to be mediated through consecutive activation of the proteases known as caspases (cysteine dependent, aspartate specific proteases) in which caspase 3 is the major effectors of apoptosis (24).

In table 3 the activity of caspase 3 was shown as fold(s) to the levels of the corresponding control group. As shown, the amount of caspase activity is significantly higher in 4 days pain group in hepatocytes, but for other comparisons there were no significant levels. It is clear that cytokines can induce oxidative stress by the generation of ROS via leakage from the mitochondrial electron transport chain (3). These inflammatory events lead to GSH depletion

Table 2. Intra-and extracellular levels of reduced and oxidized glutathione in glia of different pain groups.

Groups	Reduced glutathione (GSH) (μM)		Oxidized glutathione (GSSG)(μM)	
	Intracellular	Extracellular	Intracellular	Extracellular
Control	0.5 ± 0.04	2.4 ± 0.11	0.05 ± 0.01	2.08 ± 0.14
1 day pain induction	0.8 ± 0.05[a]	1.75 ± 0.09[a]	0.5 ± 0.06[a]	3.12 ± 0.13[a]
4 day pain induction	0.16 ± 0.03[a,b]	0.6 ± 0.04[a,b]	2.5 ± 0.1[a,b]	3.6 ± 0.18[a]
7 day pain induction	0.17 ± 0.02[a,b]	0.5 ± 0.06[a,b]	2.6 ± 0.1[a,b]	4.7 ± 0.18[a,b,c]

Values are expressed as mean ± SD and analyzed using ANOVA followed by Tukey test.
Glial cells (106 cells/ml) were prepared by dissection of hippocampus, dissociation of cells in trypsin-EDTA at 37° C and passing through a 70 and then a 25 μm nylon mesh in DMEM medium(adapted from 14).
Significant difference in comparison with a: control hepatocytes (P≤ 0.001); b: one day pain group (P≤ 0.001); c: four days pain group (P≤ 0.001), (n=10).

Table 3. Comparison of caspase activity in glia and hepatocytes of different formalin induced pain groups to their corresponding control group (folds).

Treatment / Groups	glia	hepatocyte
1 day pain induction	1.15 ± 0.12	1.26 ± 0.2
4 days pain induction	1.5 ± 0.2	1.98 ± 0.3[a]
7 days pain induction	1.5 ± 0.1	1.79 ± 0.1

Significant difference in comparison with a: 1-day pain group of glia (P≤ 0.05); b: 4-days pain group of glia (P≤ 0.05); c: control group (P≤ 0.05).
Values are expressed as mean ± SD and analyzed using ANOVA followed by Tukey test.
Glial cells (106 cells/ml) 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.
Hepatocytes (106 cells/ml) were incubated in Krebs-Henseleit buffer pH 7.4 at 37°C.

and an increases in oxidized GSH (GSSG). Cellular GSH depletion diminishes the activity of Bcl-2 protein. Bcl-2 gene products have antioxidant and antiapoptotic properties (25). So we suggest that the programmed cell death can be the major consequence of caspase activation in the 4 days pain group of hepatocytes. Our fluorescence microscope data (Photograph not shown) showed apoptotic phenotypes only in hepatocytes but not glial cells of 4 days pain, group. These data suggest that inflammatory pain, only in very intensive conditions, can generate enough ROS in liver cells [due to the activation of kupffer cells(4)] leading to MPT pore opening and enough caspase 3 activity followed by programmed cell death.

Conclusion

During the inflammatory pain in suffering rats, oxidative stress, the outcome of ROS formation occurs in glia and hepatocytes and lead to GSH depletion. In general, release of proinflammatory cytokines and oxidative stress will take effect and lead to caspase activation and programmed cell death in liver, but not the brain cells, only when the intensive induction of inflammatory pain (4 days pain group) is present.

References

(1) Watkins LR and Maier SF. Glia: a novel drug discovery target for clinical pain. *Nature Rev.* (2003) 2: 973-985

(2) Watkins LR and Maier SF. Implications of immune-to-brain communication for sickness and pain. *Proc. Natl. Acad. Sci. USA* (1999) 96: 7710-7713

(3) Rahman I and McNee W. Lung glutathione and oxidative stress: implications in cigarette smoke-induced airway disease. *Am. J. Physiol.* (1999) 277: L1067-L1088

(4) Szabo G, Romics L and Frendl G. Liver in sepsis and systemic inflammatory response syndrome. *Clin. Liver Dis.* (2002) 6: 1045-1066

(5) Arno G, Siraki A.G, Pourahmad J, Chan TS and O´Brien PJ. Endogenous and endobiotic induced reactive oxygen species formation by isolated hepatocytes. *Free Radic. Biol. Med.* (2002) 32: 2-10

(6) Skulacher VP. Mitochondrial physiology and pathology, concepts of programmed death of organells, cells and organisms. *Mol. Aspects Med.* (1999) 20: 139-184

(7) Halliwell B. Oxygen radical as key mediators in neurological disease: fact or fiction? *Ann. Neurol.* (1992) 32: S10-S15

(8) Dirks A and Leeuwenburgh C. Apoptosis in skeletal muscle with aging. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* (2002) 282: R519-R527

(9) Mayer DJ, Mao J, Holt J and Price DD. Cellular mechanisms of neuropathic pain, morphine tolerance, and their interactions. *Proc. Natl. Acad. Sci. USA.* (1999) 96: 7731-7736

(10) Preston TJ, Abadi A, Wilson L and Singh G. Mitochondrial contributions to cancer cell physiology: potential for drug development. *Adv. Drug Deliv. Rev.* (2001) 49: 45-61

(11) Nicholson DW. Caspase structure, proteolytic substrates and function during apoptosis. *Cell Death Differ.* (1999) 6: 1028-1042

(12) Nicotera P. Apoptosis and age-related disorders: role of caspase-dependent and caspase-independent pathways. *Toxicol. Lett.* (2002) 127: 189-195

(13) Vaccarino AL and Couret LC. Relationship between hypothalamic-pituitary-adrenal activity and blockade of tolerance to morphine analgesia by pain: a strain comparison. *Pain* (1995) 63: 385-389

(14) Okuda K, Sakurada C, Takahashi M, Yamada T. and Sakurada T. Characterization of nociceptive responses and spinal releases of nitric oxide metabolites and glutamate evoked by different concentrations of formalin in rats. *Pain* (2001) 92: 107-115

(15) Pourahmad J and O'Brien PJ. A comparison of hepatocyte cytotoxic mechanisms for Cu^{2+} and Cd^{2+} .*Toxicology* (2000) 143: 263-73

(16) Dermietzel R, Hertzberg EL, Kessler JA and Spray DC. Gap junctions between cultured astrocytes: immunocytochemical, molecular and electrophysiological analysis. *J. Neurosci.* (1991) 11: 1421-32

(17) Hissin J and Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal. Biochem.* (1976) 74: 214-226

(18) Shen HM, Shi CY, Shen Y and Ong CN. Detection of elevated reactive oxygen species level in cultured

rat hepatocytes treated with aflatoxin B1. *Free Radic. Biol. Med.* (1996) 21: 139-146

(19) Lykkesfeldt J, Hagen TM, Vinarsky V and Ames BN. Age-associated decline in ascorbic acid concentration, recycling, and biosynthesis in rat hepatocytes—reversal with (R)-α -lipoic acid supplementation. *FASEB* (1998) 12: 1183-1189

(20) Los M, Droge W, Stricker K, Baeuerle PA and Schulze-Osthoff K. Hydrogen peroxide as a potent activator of T lymphocyte function. *Eur. J. Immunol.* (1995) 25: 159-65

(21) Turpaev KT. Reactive oxygen species and regulation of gene expression. *Biochemistry* (2002) 67: 281-92

(22) Pourahmad J, Rezvani N, Rezaei M and Ahmadiani A. Influx and efflux of glutathione during pain induction in rat hepatocytes and glial cells. *IJP* (2006) 2: 15-19

(23) Johar D, Roth J.C, Bay G.H, Walker JN, Kroczak TJ and Los M. Inflammatory response, reactive oxygen species, programmed (necrotic like and apoptotic) cell death and cancer. *Annales Academiae Medicae Bialostocesis* (2004) 49: 31-39

(24) Friedlander RM. Apoptosis and caspases in neurodegenerative diseases. *New Engl. J. Med.* (2003) 348: 1365-75

(25) Celli A, Que GF, Gores GJ and LaRusso NF. Glutathione depletion is associated with decreased Bcl-2 expression and increased apoptosis in cholangiocytes. *Am. J. Physiol.* (1998) 275: G749-G757

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