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Inhibition of rat PC12 cell calpain activity by glutathione, oxidized glutathione and nitric oxide

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Abstract

Calpain, a calcium activated neutral protease, is involved in mediating neurotoxicity resulting from conditions of oxidative stress and free radical formation, such as hypoxia and ischemia. Nitric oxide (NO) may also be involved in modulating the cytotoxic effects of oxidative stress. We investigated the roles of reduced glutathione (GSH), oxidized glutathione (GSSG), and NO in modulating calpain activity in PC12 cells. Cell extracts were treated with GSSG, GSH, or the NO-donor S-nitroso-N-acetylpenicillamine. Calpain activity was determined by means of a fluorescent assay. Non-linear regression analysis was used to determine the type of inhibition (competitive, uncompetitive, or non-competitive). GSH displayed uncompetitive inhibition, with $K_i = 7.0 \pm 2.0$ mM (Mean ± SEM) while GSSG exhibited competitive inhibition with $K_i = 2.5 \pm 0.3$ mM. NO was an irreversible inhibitor of calpain activity. These results suggest that both GSH and GSSG may be important physiological modulators of calpain activity.

Keywords: Calpain; Glutathione; Inhibitor; Nitric oxide

Calpains are a family of calcium activated neutral cysteine proteases that serve important intracellular signalling functions. Interest in the modulation of calpain activity has been spurred by work demonstrating its involvement in mediating neurotoxic events. Previous studies show that calpains play critical roles in mediating apoptosis [1] and necrosis [9], as well as delayed cytotoxicity resulting from hypoxia [7] and ischemia [10].

Calpains exist in at least two ubiquitous forms with μ-calpain being activated by micromolar concentrations of calcium, and m-calpain requiring millimolar calcium concentrations to remain in an active form. A muscle-specific form of calpain, p94, has also been described which has intermediate calcium requirements for activation [16]. An endogenous calpain inhibitor protein, calpastatin, is also ubiquitously expressed [11].

Studies with papain-like cysteine proteases demonstrate that several intracellular regulators modulate their activity. These regulators include nitric oxide (NO) [19], reduced glutathione (GSH) and oxidized glutathione (GSSG) [4] and s-nitrosothiols [20]. Some of these regulators are known to undergo changes in their levels of expression during conditions of oxidative stress, such as during hypoxia and ischemia [17]. Given the critical role of calpains in mediating cell death, we examined whether GSH and/or GSSG or NO modulate calpain activity as a first step in understanding the mechanisms involved in oxidant-induced cytotoxicity. Several studies [4,8,19,20] investigated the roles of GSH/GSSG on calpains or closely related cysteine proteases from different cell types, but with varying results. So far no studies have focused on the effects of these putative modulators on calpain activity in rat pheochromocytoma cell lines (PC12).

Cell culture reagents were obtained from Invitrogen, Carlsbad, CA. All other reagents were obtained from Sigma, St. Louis, MO. For these experiments, PC12 cells were cultured in RPMI 1640 with L-glutamine supplemented with 5% fetal bovine serum, 10% heat-inactivated horse serum, and 50 mg/L gentamicin at 37°C, 5% CO_2. Cells were detached with 3 mM EDTA and harvested by centrifugation. Cell pellets were suspended in 400 μl extraction buffer containing 30 mM Tris (pH 6.8), 15 mM EDTA, 5 mM EGTA, 0.1 mg/ml PMSF, 0.1 mg/ml AEBSF, and 1% Triton X-100. After one cycle of freeze-thaw, the extract was centrifuged at $14\,000 \times g$ for 20 min at 4°C, and the super-

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natant used for the assays. The protein concentration of the supernatant was approximately 2 mg/ml.

Calpain-related enzymatic activity was measured using the fluorescent calpain peptide substrate N-succinyl-leu-tyr 7-amino-4-methylcoumarin as previously described [3]. Five different substrate concentrations (0, 4, 16, 48, 144 μM) and five different inhibitor concentrations (0, 0.1, 1, 5 and 10 mM) for GSH, were used for kinetic determinations. For GSSG assays, seven substrate concentrations were used (0, 20, 40, 60, 120, 180, 360 μM) and seven concentrations of GSSG (0, 0.2, 0.32, 1, 1.6, 5 and 10 mM). The reaction was initiated by the addition of 5 μl of cell extract to the wells.

Fluorescence was measured on a SpectraMAX Gemini spectrofluorometer (Molecular Devices, Sunnyvale, CA) using the SoftMax PRO software program for data capture and slope determination. Data consisting of relative fluorescence units (RFU) was obtained every 2 min for 1 h. Data was analyzed based on initial rate, defined as the slope of the linear increase of the obtained RFU value up to a maximum of 10 min. Data from $n = 7$ (GSSG and GSH) separate experiments per inhibitor was used for kinetic analysis. Each experimental point was determined as the mean of six replicates. Maximal rate was obtained in the presence of 4 mM calcium.

Steady state kinetic data were fit to a series of model equations describing competitive, non-competitive, and uncompetitive inhibition. Regression with an adaptive non-linear squares algorithm was employed for the analyses utilizing NLREG software, written by Phillip H. Sharrod [2]. Model sufficiency was evaluated based on parameter convergence with a tolerance factor of 1×10^{-10}, the overall F-value for the regression, and the magnitude and sign of the parameters obtained after convergence.

We chose the following sets of kinetic equations based on the assumption that GSH and GSSG might interact with the enzyme at the active site and would therefore inhibit product formation. To account for the fact that cell extracts obtained on different days may have contained slightly differing amounts of enzyme activity, the kinetic parameters were based on the ratio r:

$$r = \frac{V_i}{V_m}$$

where V_i is the velocity in the presence of the inhibitor and V_m is the velocity in the absence of the inhibitor. This scheme is a modification of a previously described method useful in characterizing enzyme kinetics using fluorescence-based techniques [15].

We used the following equations in our kinetic analyses to represent the competitive, uncompetitive, and non-competitive inhibition.

$$r = \frac{A + K_a}{A + K_a\left(1 + \frac{I}{K_i}\right)} \text{(Competitive Inhibition)}$$

$$r = \frac{A + K_a}{A\left(1 + \frac{I}{K_i}\right) + K_a} \text{(Uncompetitive Inhibition)}$$

$$r = \frac{A + K_a}{A\left(1 + \frac{I}{K_{ii}}\right) + K_{ia}\left(1 + \frac{I}{K_{is}}\right)} \text{(Non} - \text{competitive Inhibition)}$$

where K_a is the Michaelis–Menten constant, K_i is the dissociation constant for the enzyme-inhibitor complex, K_{ia} is the dissociation constant for the enzyme-substrate complex, and K_{ii} is the dissociation constant for the enzyme- substrate-inhibitor complex.

GSH and GSSG inhibited total calpain activity uncompetitive and competitive manners respectively, with $K_i = 7.0 \pm 2.0$ mM (Mean ± SEM) for GSH and $K_i = 2.5 \pm 0.3$ mM for GSSG (Mean ± SEM) with P-values of < 0.0001 for the non-linear regressions. Since the cell extraction procedure results in a 240-fold dilution of the cytosol volume used for enzyme assay, the maximal amount of residual GSH or GSSG in the assay system would have been <20 μM, which might have introduced a small downward bias error in the final K_i values.

It is possible that the uncompetitive inhibition of GSH might be due to an interaction with residual calpastatin. However, since both calpastatin and GSH occur in cells, it does not diminish the validity of the results, which will need to be extended to define the mechanism of inhibition.

To assure that changes in calpain activity was not due to decreases in calpain, protein extracts was treated with GSSG or GSH (10 mM) for 5 min. Protein extracts were loaded onto 8% Tris/Glycine gels (Invitrogen, Carlsbad, CA) for a total of 15 μg of protein per lane. Loading buffer was then added to the samples, which were boiled for 3 min. Gel electrophoresis was performed for 2 h at 126 V. Proteins were transferred onto nitrocellulose membranes at 26 V for 90 min. Membranes were blocked for 1 h in a casein blocking buffer, then incubated overnight in mouse anti μ-calpain and anti-m-calpain primary antibody (Affinity Bioreagents, Golden, CO) and washed and incubated with horseradish peroxidase conjugated secondary antibody (Jackson Laboratories) for 2 h. Protein bands were developed with H_2O_2 and diaminobenzidine with cobalt chloride enhancement and band density analyzed using NIH image after scanning. The results are shown in Fig. 1. Neither GSH nor GSSG at 10 mM exposure resulted in any alteration in μ-calpain or m-calpain levels, nor any change in protein mobility on the gel.

To test the effect of NO on calpain activity, the NO-donor S-nitroso- N-acetylpenicillamine (SNAP) at varying concentrations was used in the same experimental paradigm except that extracts were pre-incubated with the agent at room temperature for 5 min. The activity assays were run using low and high concentrations of calcium to preferentially activate μ-calpain or both isoforms respectively. The results of these experiments are shown in Table 1. Para-

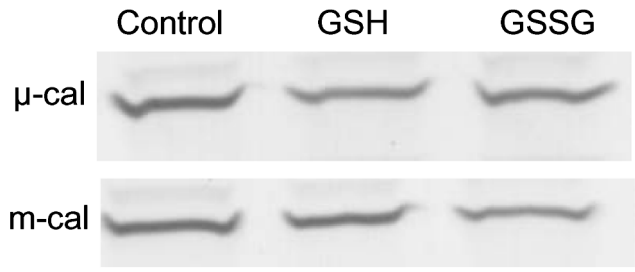

Fig. 1. Representative Western blot of μ-calpain and m-calpain after exposure of extract to 10 mM of either GSH or GSSG for 5 min. Results of density measurements from three separate experiments are as follows, expressed as density units ± SEM. Control, GSH, and GSSG, respectively: μ-calpain, 46.1 ± 2.0, 45.5 ± 1.6, and 37.0 ± 4.5; m-calpain, 49.0 ± 10.3, 44.0 ± 7.6, and 41.00 ± 8.4. No significant differences in the means were found under either experimental condition, $P > 0.21$ using parameters derived from ANOVA.

meters resulting from an ANOVA were used to test for significant differences in the means, with the significance level adjusted to $P < 0.01$ due to multiple means testing.

To assess the effect of intrinsic NO production on calpain activity, cells were treated with the non-specific NO synthase inhibitor N-methyl-L-arginine (NMLA) (1mM) and incubated for 10 min at room temperature, followed by extract preparation and assay. There was no alteration in calpain activity under these conditions, as shown in Table 1.

Both GSH and GSSG are inhibitors of calpains at concentrations found in cell cytoplasm, suggesting that the regulation of calpain activity is a complex process which may not depend solely on the endogenous inhibitor calpastatin. The current results may serve to explain why, under certain conditions, oxidative stress appears to inhibit calpain activity in calcium-permeabilized cells, [5,6] whereas in other studies increases in calpain-induced alterations in cell structure [12] and cell death occur after exposure to pro-oxidants which can be prevented by treatment with calpain inhibitors or GSH. In PC12 cells, oxidative stress is known to activate calpain activity under conditions resulting in GSH depletion, though the mechanism is unknown [13]. Taken together with the current results, one explanation for pro-oxidant mediated cell injury may be depletion of GSH and or GSSG with subsequent activation of calpain-mediated proteolysis. This in turn suggests that GSH may be playing a dual role, under some conditions protecting calpain from oxidative inhibition, and in other conditions acting as a negative modulator of activity.

SNAP-associated inhibition of calpain activity was steep and complete, occurring at concentrations between 100 nM and 1 μM. The complete abolition of any calpain activity at the higher concentration of the agent, the maintenance of inhibition at assay time points as long as 1 h after addition of the agent, well after any additional NO production by the agent should have ceased, and the lack of effect of increasing calcium concentrations on calpain activity suggests that NO-mediated inhibition under these conditions was not reversible. The current results support previous work providing evidence for NO-mediated irreversible inhibition of m-calpain [8].

To determine if basal production of NO might have altered calpain activity, cells were treated with an NO synthase inhibitor prior to extraction. Since extract obtained from these cells had the same calcium-stimulated calpain activity as untreated cells, we conclude that basal NO does not significantly alter calpain activity within the resolution limits of the assay.

Levels of GSH and GSSG in rat neurons and glia have been estimated to be in the range of 2.5 mM and 3.8 mM respectively [14]. The current results suggest that under physiological conditions, GSH and GSSG may play a role in modulating calpain activity, while basal NO production is not involved in this modulation. GSH, and to a lesser extent GSSG may also function as reversible inhibitors of other proteinases [18]. This introduces the possibility that GSH and GSSG may play a role in modulating not only calpain-associated protease activity, but also the activity of non-cysteinyl proteases.

Table 1
Effects of SNAP and NMLA on Calpain Activity[a]

	Calcium (mM)		
SNAP log(nM)	0.00	0.14	1.40
C (0)	11859 (428)	26216 (990)	33948 (885)
1	8950 (374)	12174 (832)*	18480 (2384)*
2	8780 (423)	14173 (1526)*	18601 (2380)*
3	9925 (322)	8304 (267)*	9591 (350)*
4	8472 (243)	8381 (192)*	10566 (248)*
NMLA 1 mM	10850 (735)	27103 (720)	33055 (1575)

[a] Data expressed as V_{max} (RFU/min). *Significantly different from control, $P < 0.01$ $n = 4$–6.

[1] Blomgren, K., Zhu, C., Wang, X., Karlsson, J.O., Leverin, A.L., Bhar, B.A., Mallard, C. and Hagberg, H., Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia-ischemia: a mechanism of "pathological apoptosis" J. Biol. Chem., 276 (2001) 10191–10198.
[2] Dennis, J.E., Gay, D.M. and Welsch, R.E., An adaptive nonlinear squares algorithm, ACM Trans. Math. Software, 7 (1981) 3–7.
[3] DePetrillo, P.B., Calcium-activated neutral protease activity is decreased in PC12 cells after ethanol exposure, J. Neurochem., 68 (1997) 1863–1869.
[4] Di Cola, D. and Sacchetta, P., Irreversible inactivation of calcium-dependent proteinases from rat liver by biological disulfides, FEBS Lett., 210 (1987) 81–84.
[5] Guttmann, R.P., Elce, J.S., Bell, P.D., Isbell, J.C. and Johnson, G.V., Oxidation inhibits substrate proteolysis by calpain I but not autolysis, J. Biol. Chem., 272 (1997) 2005–2012.
[6] Guttmann, R.P. and Johnson, G.V., Oxidative stress inhibits calpain activity in situ, J. Biol. Chem., 273 (1998) 13331–13338.
[7] Jiang, Q. and Stys, P.K., Calpain inhibitors confer biochemical, but not electrophysiological, protection against anoxia in rat optic nerves, J. Neurochem., 74 (2000) 2101–2107.

[8] Koh, T.J. and Tidball, J.G., Nitric oxide inhibits calpain-mediated proteolysis of talin in skeletal muscle cells, Am. J. Physiol. Cell. Physiol., 279 (2000) C806–C812.
[9] Kohli, V., Madden, J.F., Bentley, R.C. and Clavien, P.A., Calpain mediates ischemic injury of the liver through modulation of apoptosis and necrosis, Gastroenterology, 116 (1999) 168–178.
[10] Li, P.A., Howlett, W., He, Q.P., Miyashita, H., Siddiqui, M. and Shuaib, A., Postischemic treatment with calpain inhibitor MDL 28170 ameliorates brain damage in a gerbil model of global ischemia, Neurosci. Lett., 247 (1998) 17–20.
[11] Maki, M., Ma, H., Takano, E., Adachi, Y., Lee, W.J., Hatanaka, M. and Murachi, T., Calpastatins: biochemical and molecular biological studies, Biomed. Biochim. Acta, 50 (1991) 509–516.
[12] Nakamura, Y., Fukiage, C., Azuma, M. and Shearer, T.R., Oxidation enhances calpain-induced turbidity in young rat lenses, Curr. Eye Res., 19 (1999) 33–40.
[13] Ray, S.K., Fidan, M., Nowak, M.W., Wilford, G.G., Hogan, E.L. and Banik, N.L., Oxidative stress and Ca2 + influx upregulate calpain and induce apoptosis in PC12 cells, Brain Res., 852 (2000) 326–334.
[14] Rice, M.E. and Russo-Menna, I., Differential compartmentalization of brain ascorbate and glutathione between neurons and glia, Neuroscience, 82 (1998) 1213–1223.
[15] Sem, D.S. and McNeeley, P.A., Application of fluorescence polarization to the steady-state enzyme kinetic analysis of calpain II, FEBS Lett., 443 (1999) 17–19.
[16] Sorimachi, H., Ishiura, S. and Suzuki, K., Structure and physiological function of calpains, Biochem. J., 328 (1997) 721–732.
[17] Tyurin, V.A., Tyurina, Y.Y., Borisenko, G.G., Sokolova, T.V., Ritov, V.B., Quinn, P.J., Rose, M., Kochanek, P., Graham, S.H. and Kagan, V.E., Oxidative stress following traumatic brain injury in rats: quantitation of biomarkers and detection of free radical intermediates, J. Neurochem., 75 (2000) 2178–2789.
[18] Upadhya, G.A. and Strasberg, S.M., Glutathione, lactobionate, and histidine: cryptic inhibitors of matrix metalloproteinases contained in University of Wisconsin and histidine/tryptophan/ketoglutarate liver preservation solutions, Hepatology, 31 (2000) 1115–1122.
[19] Venturini, G., Salvati, L., Muolo, M., Colasanti, M., Gradoni, L. and Ascenzi, P., Nitric oxide inhibits cruzipain, the major papain-like cysteine proteinase from Trypanosoma cruzi, Biochem. Biophys. Res. Commun., 270 (2000) 437–441.
[20] Xian, M., Chen, X., Liu, Z., Wang, K. and Wang, P.G., Inhibition of papain by S-nitrosothiols. Formation of mixed disulfides, J. Biol. Chem., 275 (2000) 20467–20473.