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EFFECTS OF CALPAIN ON ANTIOXIDANT ENZYME ACTIVITIES

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The activities of superoxide dismutase, catalase and glutathione reductase were not affected by *in vitro* incubation with the intracellular proteinase calpain, suggesting that these enzymes are not *in vivo* substrates of calpain. In contrast, the activity of another important antioxidant enzyme, glutathione peroxidase, is stimulated *in vitro* by calpain. This may explain the correlation between elevations in glutathione peroxidase activity and calpain activity which occur in aging, exercised and dystrophic muscle. Calpain treatment *in vitro* caused a large decrease in the activity of carnosine synthetase which is involved in the synthesis of the putative antioxidant carnosine. This may be the reason for the *in vivo* correlation between elevated calpain and diminished carnosine levels in aging, hypertensive, denervated and dystrophic muscles.

KEY WORDS: Calpain, calcium-activated neutral proteinase, glutathione peroxidase, carnosine synthetase.

INTRODUCTION

It is now recognized that aerobic organisms have evolved elaborate defense mechanisms to protect themselves from possible damage caused by the generation of reactive oxygen species and free radicals which may be produced as a result of the use of oxygen as a terminal electron acceptor.[1] Such mechanisms involve enzymes which can convert the reactive species into inert compounds, and scavenger antioxidants which can non-enzymatically eliminate the reactive species. The most important enzymes which appear to have primary antioxidant functions are superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, and attention has been focussed on the variations in the levels of these enzymes as a result of altered metabolic states including disease.[2–6] Scavenger antioxidants are structurally very diverse and include both lipophilic and hydrophilic compounds.[7] Recent studies indicate that the histidine dipeptides such as carnosine and anserine are antioxidants,[8] and it has been suggested that these compounds may have particularly significant antioxidant roles in muscle and nervous tissue where they may be present at relatively high and variable levels.[9]

It is possible that intracellular proteolysis of the antioxidant enzymes may play a significant role in establishing different steady state levels of these enzymes, and similarly, intracellular proteolysis could also be a factor in the control of scavenger antioxidant levels as a result of intracellular proteolysis of the enzymes involved in

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the synthesis and degradation of the dipeptides. A group of very widely distributed intracellular proteinases which have been implicated in the intracellular degradation and turnover of a number of proteins are the calcium-activated cysteine proteinases (EC 3.4.22.17), generally referred to as calpains.[10] Two types of calpains exist, calpains I and II, which have respective requirements for micro- and millimolar levels of Ca^{2+} ions *in vitro* for full activity. The proteolytic specificities of calpains I and II are essentially identical, and although the identities of amino acid residues adjacent to the scissile peptide bond are somewhat variable, there appears to be a preference for a basic or bulky side chain at the P_1 position and a hydrophobic side chain at the P_2 position.[10] Calpains have been reported to degrade a number of contractile and cytoskeletal proteins (including myosin light chain, tropomyosin, vimentin, tubulin, spectrin and filamin), cytoplasmic and membrane-bound enzymes such as protein kinases, transglutaminases, hydroxylases and also protein receptors including those for various steroids and growth factors.[10] To date however, there have been no reports which have linked calpain-mediated proteolysis to possible changes in the levels of antioxidant enzymes and compounds. In the only previous study on possible calpain involvement in antioxidant metabolism, it was concluded that the proteolytic conversion of xanthine dehydrogenase to the antioxidant enzyme xanthine oxidase could not be effected by calpain.[11]

In the present studies, in order to examine in more detail the possible role for calpains in antioxidant metabolism, we have investigated the *in vitro* effects of a calpain preparation on four antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) and we have also studied the effects of calpain on carnosine synthetase, the enzyme involved in histidine dipeptide biosynthesis.[8] These studies have revealed that calpain treatment of glutathione peroxidase (EC 1.11.1.9) and carnosine synthetase (EC 6.3.2.11) significantly alters the activities of these enzymes, and the results suggest that *in vivo* levels of calpain activity may play a role in tissue antioxidant metabolism.

MATERIALS AND METHODS

Materials

Reagents and enzymes, including bovine kidney superoxide dismutase (S-2139), bovine liver catalase (C-40), bovine erythrocyte glutathione peroxidase (G-6137) and yeast glutathione reductase (G-4759), were purchased from Sigma Chemical Co. Fresh chicken gizzards used for calpain preparation were purchased from a local poultry supplier.

Enzyme Preparations

Calpain II from chicken gizzard smooth muscle was prepared as described previously.[12] Carnosine synthetase activity in rat cardiac muscle was recovered in the supernatant fraction obtained by centrifugation at 3000 g for 5 min at 4° following pulverization and homogenization[13] of the tissue in 15 vols of 50 mM Tris, pH 7.2.

Enzyme Assays

Standard methods of assay were used for the following antioxidant enzymes. Superoxide dismutase activity was measured by the procedure of Elstner and Heupel[14] in which activity is calculated from the degree of inhibition of nitrite formation from hydroxylammonium chloride in the presence of xanthine and xanthine oxidase. Catalase activity was measured by analysis of the rate of hydrogen peroxide decomposition using the spectrophotometric procedure of Aebi.[15] Glutathione peroxidase activity was measured using *t*-butyl hydroperoxide and glutathione as substrates in the glutathione reductase-linked "continuous monitoring" procedure of Flohe and Gunzler.[16] Glutathione reductase activity was determined by measurement of the rate of NADPH oxidation in the presence of oxidized glutathione.[17]

For assay of carnosine synthetase using non-radioactive β-alanine, the muscle preparation (150 μl) was incubated in a final volume of 0.5 ml containing 3 mM histidine, 4 mM Mg-ATP, 30 mM β-alanine, and 2 mg/ml bovine serum albumin[18] for 1 h at 37°, after which the concentration of β-alanine in a deproteinized sample of the incubation mixture was determined on a Beckman 119CL Amino Acid Analyzer. Control experiments with tissue incubated in the absence of added substrates showed that negligible amounts of β-alanine were introduced into the assay from the tissue homogenate. Activity levels of the enzyme were calculated from the amounts of β-alanine used during the incubation with substrates, and the muscle preparations had specific activity levels of approximately 12 nmol β-alanine used/min/mg protein.

Calpain was used to pretreat each of the above enzyme preparations at various calpain:enzyme ratios in the presence of 2 mM Ca^{2+} in 10 mM bistrispropane pH 8.0 for 2 h at 30°, after which the calpain activity was inhibited by the addition of the calpain inhibitor E-64.[19] Calpain activity was measured in these experiments by the azo-casein hydrolysis procedure described previously.[20] Aliquots of these solutions were then used for assay of each enzyme, and the numbers of units of enzyme activity used in each assay were 0.5 (superoxide dismutase), 4 (catalase), 0.02 (glutathione peroxidase), 0.003 (glutathione reductase), and 2 (carnosine synthetase). Control experiments were performed to establish that exposure to the preincubation procedure in the absence of active calpain did not significantly affect the enzyme activities.

Statistical Analysis

Mean values ± SD were calculated for each calpain : enzyme ratio used in six different experiments. Student's *t* test was used to evaluate significant differences between data sets based on the confidence level (P value) calculated for the difference, and values for $P > 0.05$ were taken to indicate no significant difference between data sets.

RESULTS AND DISCUSSION

Effect of Calpain on the Activities of Superoxide Dismutase, Catalase, and Glutathione Reductase

In the cases of these three enzymes, incubation with calpain at weight ratios of calpain: enzyme of up to 10:1 indicated that calpain did not affect the activity of

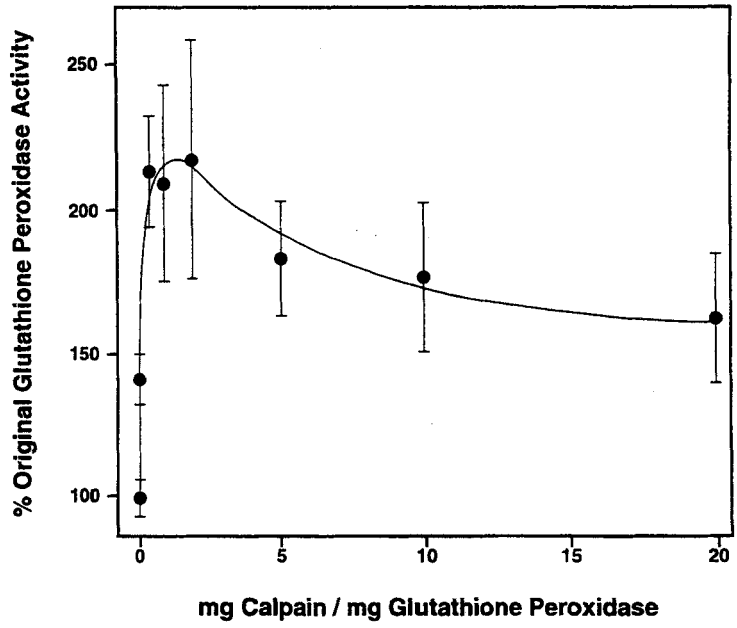

FIGURE 1 The effect of calpain treatment on the activity of erythrocyte glutathione peroxidase. Glutathione peroxidase was incubated with different amounts of calpain, after which the calpain was inactivated and the glutathione peroxidase activity was measured as described in the Methods. Results are plotted relative to the activity of glutathione peroxidase put through the procedure in the absence of calpain. Bar lines show the standard deviation for each set of determinations.

the enzymes (results not shown). The activities of the enzymes pretreated with calpain were within 10% of the control values, and these differences were found to be statistically insignificant. These results therefore indicate that these enzymes are not *in vitro* substrates of calpains and that changes in tissue calpain activity levels probably do not affect the activities of these enzymes.

Effect of Calpain on Glutathione Peroxidase Activity

As shown in Figure 1, pretreatment of glutathione peroxidase with calpain resulted in a statistically-significant specific activity increase of glutathione peroxidase with a maximum activation of around two-fold at a calpain:enzyme weight ratio of about 1:1. When the calpain:enzyme ratio was increased to as high as 20:1, the enzyme still displayed an activation effect of about 1.6 times that of the control activity. These results indicate that glutathione peroxidase is susceptible to proteolysis by calpain at high ratios of calpain:glutathione peroxidase and that the partial proteolysis which occurs results in an increase in the specific activity of the peroxidase. The peroxidase appears to be resistant to further proteolysis which causes activity loss as the enzyme was still active after treatment with calpain at a calpain:enzyme ratio of 20:1.

Inspection of the amino acid sequence of bovine erythrocyte glutathione peroxidase[21] indicates that there are a number of possible cleavage sites for calpains based on the known preferences for amino acid residue types at the P_1 and P_2 positions relative to the scissile bond.[10] However, many of these potential sites

for calpain-mediated proteolysis may be unavailable for proteolysis, as recent studies[22] have indicated that glutathione peroxidase may be folded into a globular core which is resistant to proteolysis, with only an exposed N-terminal region of 17 residues that is susceptible to proteolysis. It is therefore possible that this N-terminal sequence, which contains potential sites for calpain cleavage, may be the site of the *in vitro* calpain-mediated proteolysis. Further studies will be needed to identify the site(s) of cleavage in glutathione peroxidase by calpain to examine this possibility and to determine if resistance of the enzyme to further proteolysis (as evidenced by retention of catalytic activity in the presence of high levels of calpain) is a reflection of the existence of a central core region which is resistant to calpain-mediated proteolysis.

The results from this study suggest the possibility that *in vivo* levels of glutathione peroxidase activity may be modulated by calpain activity where there is a relatively high level of calpain activity in relation to glutathione peroxidase activity or where the enzyme is continuously exposed to calpain activity. In these cases, calpain activation caused by changes in Ca^{2+}, calpastatin or calpain activator levels[9,23] could cause significant increases in the specific activity of the tissue glutathione peroxidase which would enable the tissue to have a more effective antioxidant defense system. In this context, it is interesting to note that calpain levels have been observed to increase in skeletal muscles as a result of aging,[13] exercise[24] and dystrophy,[25] and other studies have reported increases in glutathione peroxidase activity in skeletal muscles also as a result of aging,[3] exercise[5,6] and dystrophy.[2] The results of the present study together with the reports of parallel activity changes of calpain and glutathione peroxidase in skeletal muscle under different circumstances therefore are suggestive that calpain may have an *in vivo* role in antioxidant metabolism through alteration of glutathione peroxidase activity. In order to establish that changes in glutathione peroxidase activity in tissues are a direct consequence of calpain-mediated proteolysis, it will be necessary to determine if such modifications can occur *in vivo* to glutathione peroxidase in aging, exercised or dystrophic muscles.

Effect of Calpain on Carnosine Synthetase Activity

Cardiac muscle preparations were preincubated with calpain at calpain:tissue protein weight ratios of up to 0.17. As shown in Figure 2, there was a small but statistically significant increase in the activity of the enzyme at the lower ratios of calpain:carnosine synthetase used, but at higher ratios, there was a significant decrease in carnosine synthetase activity down to a level of about 40% of the control (undigested) activity.

These results indicate that although limited proteolysis of carnosine synthetase by calpain causes a small increase in the activity of the enzyme, further cleavage of the enzyme by calpain causes a large decrease in activity, which may be indicative of extensive proteolytic degradation of the enzyme. Discussion of the possible sites of proteolysis in this enzyme must await further studies on the proteolytic fragments of carnosine synthetase, and details of the primary and tertiary structure of the enzyme which are currently unknown.

In terms of carnosine levels in muscle, calpain activation would only be expected to affect carnosine biosynthesis as muscle contains carnosine synthetase but little or no carnosinase activity.[8] Previous studies have shown that muscle carnosine levels are decreased as a result of aging,[9] hypertension,[9] dystrophy[8] and denervation,[8] although such changes were not directly ascribed to changes in carnosine

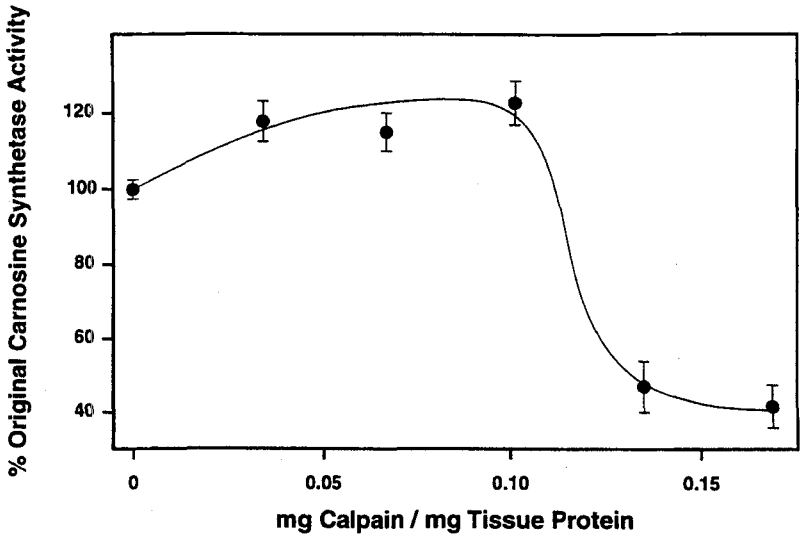

FIGURE 2 The effect of calpain treatment on the activity of carnosine synthetase from cardiac muscle. The carnosine synthetase preparation was incubated with different amounts of calpain, after which the calpain was inactivated and the carnosine synthetase activity was measured as described in the Methods. Results are plotted relative to the activity of the carnosine synthetase preparation put through the procedure in the absence of calpain. Bar lines show the standard deviation for each set of determinations.

synthetase activity. However, as calpain activities or titers in muscle have been reported to increase in aging,[13] hypertension,[13] dystrophy,[25] and denervation,[26] the *in vivo* reciprocal relationship between muscle carnosine levels and calpain activity could be explained by the observation that decreased activity of carnosine synthetase is observed *in vitro* at high calpain:carnosine synthetase ratios. This inverse correlation does not appear to exist however as a consequence of exercise, when there appears to be little change in muscle carnosine levels[27] even though increases in calpain activity have been reported.[24] An analysis of the extent of calpain-mediated proteolytic degradation of carnosine synthetase in muscles will be necessary to determine if calpain is involved in the *in vivo* degradation of carnosine synthetase and to explain why the exercised state appears to follow a different pattern from that observed for carnosine levels in the other conditions.

In conclusion, the present *in vitro* studies have shown that calpain can cause a significant activation of glutathione peroxidase and an inhibition of carnosine synthetase. These results correlate with previously reported *in vivo* changes in the activities of calpain and glutathione peroxidase and of carnosine levels, and suggest that calpain-mediated proteolysis of the two enzymes may occur *in vivo*, thereby affecting normal antioxidant metabolism in certain tissues.

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