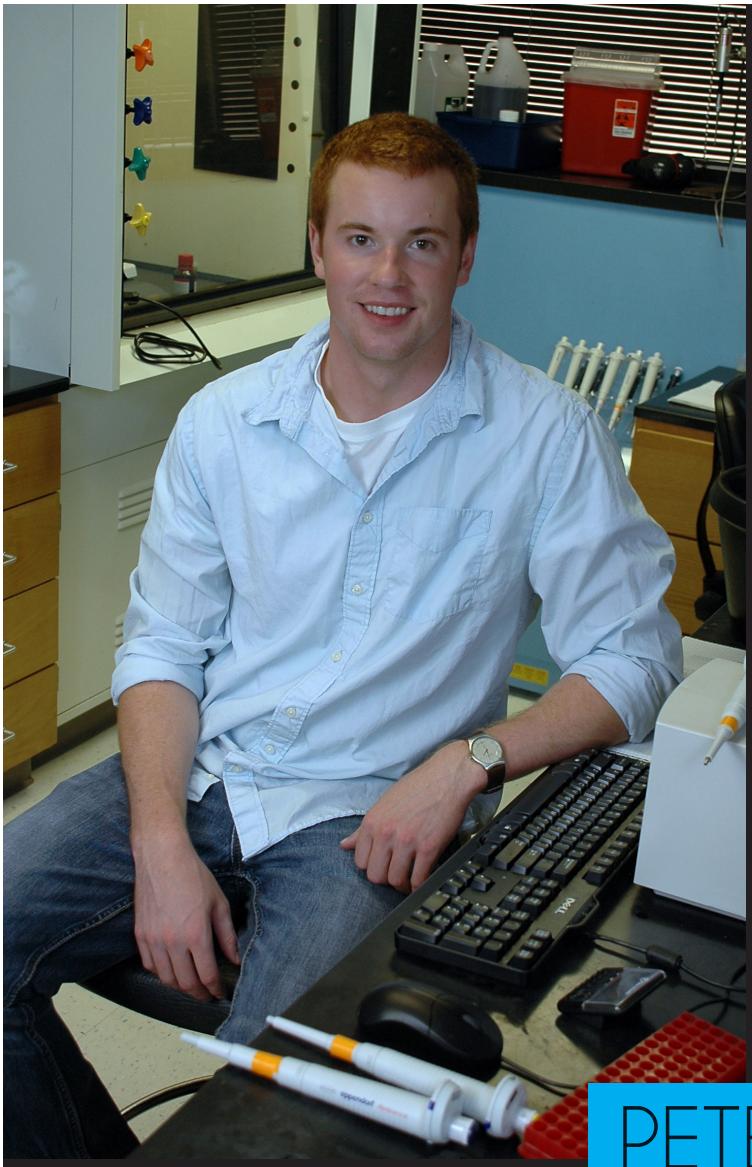

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FINAL RESEARCH MANUSCRIPTS

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Calcium Stimulated Glutathionylation of α-Ketoglutarate Dehydrogenase: Fine-Tuning a Protective Antioxidant Response

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ABSTRACT

The enzyme α-ketoglutarate dehydrogenase (KGDH) plays a predominant role in mitochondrial function as it controls the flux of substrates through the Krebs Cycle and provides reducing equivalents crucial for oxidative phosphorylation. Previous studies have shown that KGDH is reversibly inhibited in response to an increase in mitochondrial free radical production. Inhibition is due to the formation of a mixed disulfide between the covalently bound cofactor of KGDH, lipoic acid, and glutathione (glutathionylation). This protects the enzyme against irreversible oxidative damage and reduces free radical production. It is therefore critical that factors that regulate the reversible glutathionylation of KGDH be identified. Ca2+ is a potent activator of KGDH. Yet, if present in excessive amounts, Ca2+ can induce mitochondrial free radical production and induce programmed cell death, termed apoptosis. Because of these implications, this study aimed to determine if Ca2+ plays a modulatory role in glutathionylation of KGDH. Mitochondria from rat heart were treated with H2O2 and different concentrations of Ca2+ for various periods of time and KGDH activity was analyzed. Results revealed that in the absence of Ca2+, there was lag period in the glutathionylation and inhibition of KGDH. However, treatment with Ca2+ eliminated this period of lag and increased the rate of inhibition. Data suggests that Ca2+ increases the half-life of the reduced sulfhydryl groups on the lipoic acid cofactor, increasing the likelihood for glutathionylation. Thus, Ca2+ effectively primes lipoic acid for reversible modification. Cardiovascular disease is often associated with disruption in the normal flux of Ca2+ ions across the mitochondrial membrane leading to Ca2+ overload, free radical production, and the induction of apoptosis. Further study may show that Ca2+-stimulated redox-dependent inhibition of KGDH is in fact a response to prevent cell death.

INTRODUCTION

KGDH is an enzyme complex that consists of multiple copies of three subunits that together catalyze the conversion α-ketoglutarate into succinyl-CoA (Scheme 1).[10] This process produces reducing equivalents, in the form of NADH, necessary for the production of ATP by oxidative phosphorylation. The overall reaction catalyzed by KGDH serves as a key regulatory site within the Krebs Cycle making its activity directly related to the amount of reducing equivalents available for the production of ATP.[4,6] In effect, KGDH controls the production of ATP. This property demonstrates the importance of KGDH and merits investigation into its regulation.

Previous studies have shown that KGDH is reversibly inhibited in the presence of free radicals or the pro-oxidant H_2O_2.[1,7,8] This process involves the formation of a mixed disulfide between its covalently bound lipoic acid cofactor and glutathione, termed glutathionylation.[1,7] Upon removal of free radicals and pro-oxidants, glutathione is removed by the enzyme glutaredoxin, after which KGDH activity is restored (Scheme 2). Glutathionylation can be seen as a protective mechanism, as it limits KGDH activity, and thus the supply of electrons in the form of NADH to the electron transport chain, a source of radical species.[2,3] Thus, down regulation of KGDH limits the amount of free radicals within the mitochondrial environment. Additionally, glutathionylation prevents the reactive sulfhydryl groups on lipoic acid from oxidative damage, which could result in enzyme inactivation.[1]

Ca^{2+} has been found to activate KGDH by increasing its affinity for NAD^+ and α-ketoglutarate, its two substrates.[5] Yet, if present in excessive amounts, Ca^2+ can initiate programmed cell death, known as apoptosis.[9] An overabundance of Ca^{2+} within the mitochondria causes swelling and eventual rupture of its outer membrane. As a result cytochrome c is released, which is the first step in a series of events that ends in cell death.[9] Since KGDH helps to maintain the electrochemical gradient that creates Ca^{2+} influx, its regulation could be an important factor in events leading to apoptosis. This study sought to examine the combined effects of Ca^{2+} and H_2O_2 on the regulation of KGDH through glutathionylation, as well as its potential role in the process of cell death.

MATERIALS AND METHODS

Isolation of Mitochondria from Rat Heart. Sprague-Dawley rats were decapitated, after which the heart was excised and then perfused with isolation buffer (210 mM mannitol, 70 mM sucrose, 10 mM MOPS, 1.0 mM EDTA, pH 7.4) to displace blood. Hearts were then minced and homogenized using a Polytron homogenizer. The homogenate was centrifuged at 500 x g for 5 min (4°C). The supernatant was then filtered with cheesecloth and centrifuged at 10000 x g for 10 min (4°C). The mitochondrial pellet was then resuspended with ice-cold isolation buffer to a final concentration of 25 mg/ml. Protein determinations were made employing the BCA method, using BSA as the standard.

Incubation of MItochondria with H_2O_2 and/or Ca^{2+}. Mitochondria were diluted to 0.5 mg/ml in buffer (210 mM mannitol, 70 mM sucrose, 10 mM MOPS, 5.0 mM K2HPO4, pH 7.4). Respiration was initiated upon the addition of 5.0 mM α-ketoglutarate and allowed to continue for 1.0 min at which point varying concentrations of Ca^{2+} were added. After 1.0 min, to allow for Ca^{2+} uptake by the mitochondria, H_2O_2 (25 μM) was added. At distinct time points, Triton X-100 was added to a final concentration of 0.05% effectively preventing further inhibition or reactivation of KGDH. Samples were then prepared for analysis of KGDH activity or the glutathionylation status or oxidation state of lipoic acid.

Assay for α-Ketoglutarate Dehydrogenase Activity. Mitochondria were diluted to 0.05

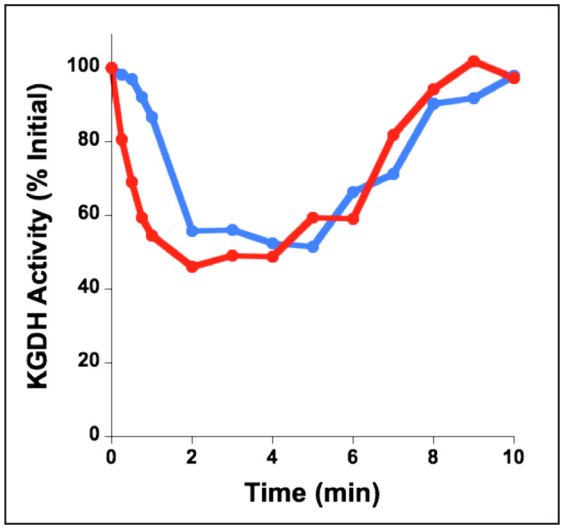


Figure 1. Reversible Inhibition of KGDH in Response to H_2O_2 and Ca^{2+}. Isolated rat heart mitochondria (0.5 mg/ml) were allowed to respire upon the addition of 5.0 mM α-ketoglutarate. After 1 min, 0 µM or 20 µM Ca^{2+} was added. One minute after the addition of Ca^{2+}, 25 µM H_2O_2 was added. At the specified time points, mitochondria were solubilized in 0.05% Trition X-100. KGDH activity was then measured spectrophotometrically.

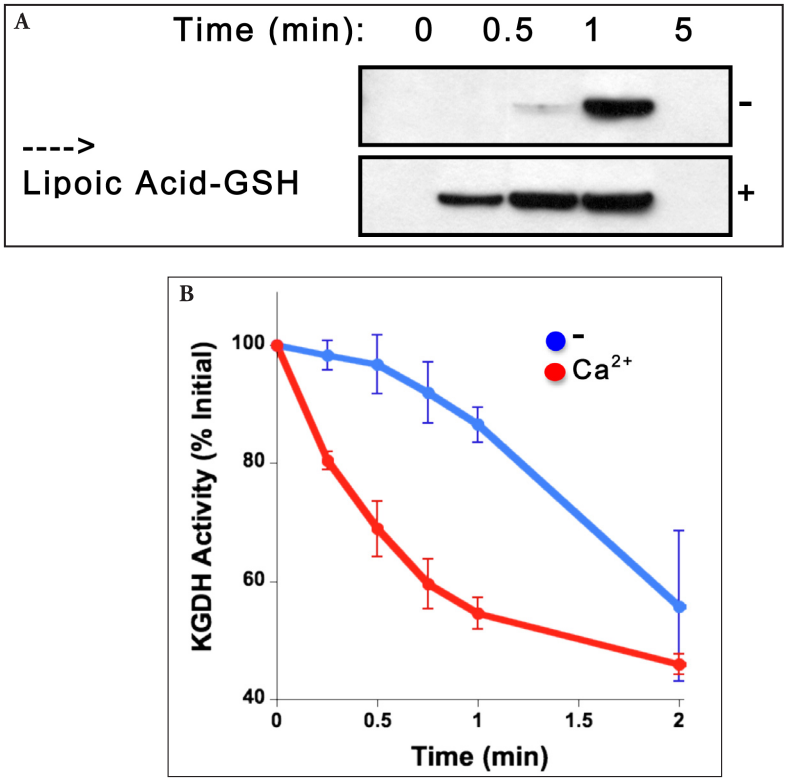


Figure 2. Reversible Glutathionylation of KGDH in Response to H2O2 and Ca^{2+}. A) Isolated rat heart mitochondria (0.5 mg/ml) were allowed to respire upon the addition of 5.0 mM α-ketoglutarate. After 1 min, 0 µM or 20 µM Ca^{2+} was added. One minute after the addition of Ca^{2+}, 25 µM H_2O_2 was added. At the specified time points, samples were evaluated by Western blot analysis using antibody to glutathionylated lipoic acid. B) Initial response of KGDH to H_2O_2 in the presence and absence of Ca^{2+} (t = 0 to 2 min from Fig. 1).

mg/ml in 25 mM MOPS, 0.05% Triton X-100, pH 7.4. KGDH activity was assayed spectrophotometrically as the rate of NAD^+ reduction to NADH at 340nm (ε = 6.2 M 1•cm-1) upon the addition of 5.0 mM MgCl2, 2.5 mM α-ketoglutarate, 0.1 mM CoASH, 0.2 mM thiamine pyrophosphate, and 1.0 mM NAD+.

Western Blot Analysis. Mitochondrial protein was suspended in 27 mM Tris-HCl, 35.2 mM EDTA, 0.55 mM SERVA Blue G250, 0.05 mM phenol red, and 100 mM iodoacetamide at pH 8.5. Protein was resolved on a 10% SDS-PAGE gel and electrotransferred onto nitrocellulose membrane. Membrane immobilized protein were analyzed utilizing polyclonal antibodies specific to the E2 subunit of KGDH, lipoic acid, or glutathione. Primary antibody binding was visualized utilizing peroxidase-conjugated secondary antibody and chemiluminescent substrate.

RESULTS

Previous studies have shown that KGDH is reversibly inhibited in the presence of H_2O_2.[1,7,8] This inhibition was linked to reversible glutathionylation of the lipoic acid cofactor on the E2 subunit of KGDH.[1,7] Experiments were performed to determine the role of Ca^{2+} on this process. Isolated rat heart mitochondria (0.5 mg/ml) respiring on α-ketoglutarate (5.0 mM) were treated with 25 µM H2O2 and 0 or 20 µM Ca^{2+}. At specified times, mitochondria were disrupted and KGDH activity was measured. As shown in Figure 1, treatment of mitochondria with H_2O_2 in the absence of Ca^{2+} resulted in a 50% decline in KGDH activity within 2 min followed by full recovery at 10 min. However, there was a ~45 s lag period after the addition of H_2O_2 prior to significant inhibition. The addition of 20 µM Ca^{2+} to the mitochondrial incubation eliminated this lag period and increased the rate of H_2O_2-induced inhibition of KGDH. The presence of Ca^{2+} did not appear to affect the degree of inhibition or the rate of recovery. A Ca^{2+} concentration of 20 µM produced the greatest amount of inhibition in the presence of H_2O_2. Additionally, physiologically relevant concentrations of Mg^{2+} were tested in a similar manner with no effect (results not shown). Thus, the effect of Ca^{2+} appears specific.

Experiments were performed to determine if the increased rate of inhibition in the presence of Ca^{2+} was due to glutathionylation. Western blot analysis revealed that the increase in the rate of KGDH inhibition in the presence of Ca^{2+} corresponded to an increase in glutathionylation of the covalently bound lipoic acid cofactor of E2 of KGDH (Figure 2A). Glutathionylation of KGDH in mitochondria treated with and without Ca^{2+} only differed within the first minute following the addition of H_2O_2. As shown in Figure 2B, this is reflective of the Ca^{2+} stimulated elimination of the lag period for H_2O_2-mediated inhibition of KGDH.

Lipoic acid, a cofactor covalently linked to the E2 subunit of KGDH and required for activity, is the site of glutathionylation. During enzymatic catalysis lipoic acid cycles between the reduced and oxidized state. For glutathionylation to occur lipoic acid must be in the reduced state. We hypothesized that Ca^{2+} mediates its effects on H_2O_2-induced KGDH inhibition by increasing the half-life of reduced sulfhydryl groups on lipoic acid. To test this, KGDH was derivatized with iodoacetamide (100 mM), a sulfhydryl reactive compound that will react with reduced lipoic acid but not the oxidized form

(Scheme 3), prior to Western Blot analysis. Analysis was performed with an antibody that recognizes native lipoic acid but not idoacetamide derivatized lipoic acid. If our hypothesis was correct, we would expect to see a decrease in antibody binding upon treatment with Ca^{2+}. As shown in Figure 3, with the addition of α-ketoglutarate, the amount of reduced lipoic acid (iodoacetamide reactive) increased relative to mitochondria incubated in the absence of α-ketoglutarate or with Ca^{2+} alone. In the presence of both Ca^{2+} and α-ketoglutarate, almost all of the lipoic acid present was in the reduced state and thus primed for glutathionylation.

DISCUSSION

Efforts to determine the effects of Ca^{2+} on KGDH activity are essential in completely understanding the roles of this important enzyme. The results of this study have shown that Ca^{2+} effectively primes the lipoic acid cofactor of KGDH for H2O2-induced glutathionylation. Ca^{2+} is a known activator of KGDH, increasing the rate of the reaction occurring in the E1 subunit.[5] This causes the reaction catalyzed by E3 to be the rate-limiting step. As a result, the half-life of the reduced sulfhydryl groups on lipoic acid present on the E2 subunit are increased, giving lipoic acid a stronger likelihood of being glutathyionylated.

The lag period exhibited in H_2O_2 treatments in the absence of Ca^{2+} may be representative of the time needed for molecular interactions to occur, suggesting that glutathionylation is an enzyme catalyzed process. Further, efforts to reconstitute glutathionylation utilizing the purified enzyme were unsuccessful, even in the presence of Ca^{2+} (data not shown). This data also supports the argument that upon treatment of mitochondria with H_2O_2 glutathionylation and inhibition of KGDH requires a yet to be discovered enzyme. The ability of Ca^{2+} to reduce the lag period for inhibition suggests that, in addition to increasing the half-life of the reduced lipoic acid and thus the rate of glutathionylation, Ca^{2+} may aid the association of KGDH with the glutathionylating enzyme.

Ca^{2+} has differing roles in mitochondria. It activates KGDH that increases the rate of ATP production. Paradoxically, Ca^{2+} can also induce mitochondria free radical production and cell death if present in high concentrations within the mitochondria.[5,9] Mitochondrial Ca^{2+} uptake requires a membrane potential. KGDH plays a significant role in maintaining the electrochemical proton gradient across the mitochondrial membrane that is necessary for

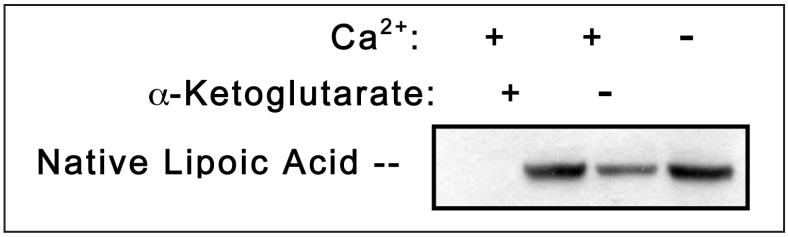
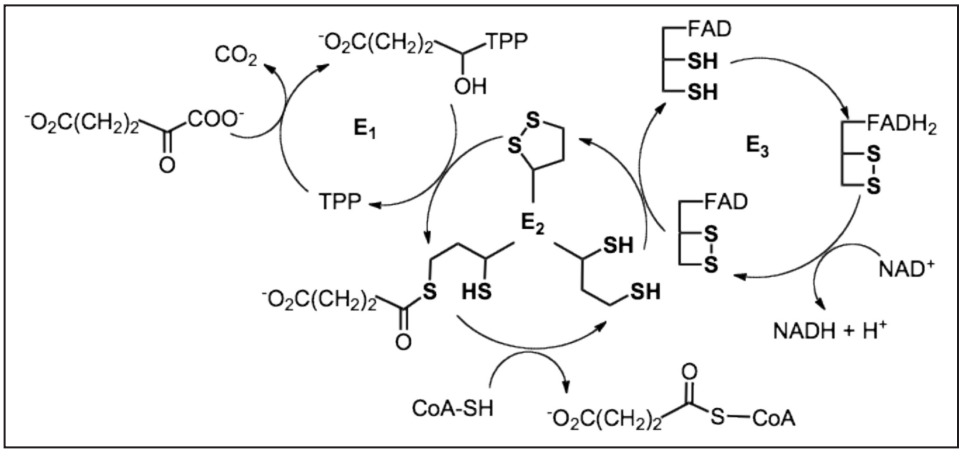
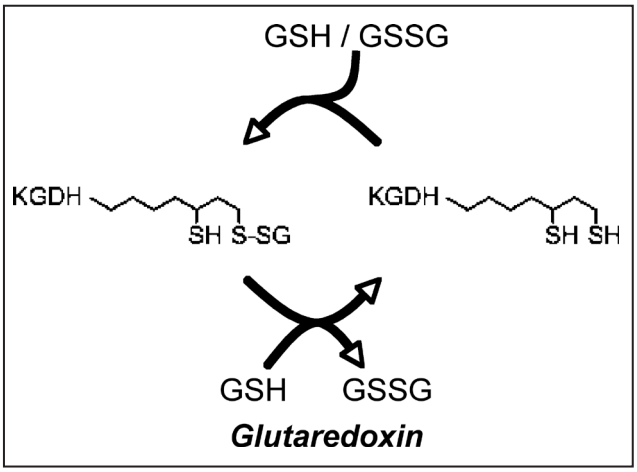


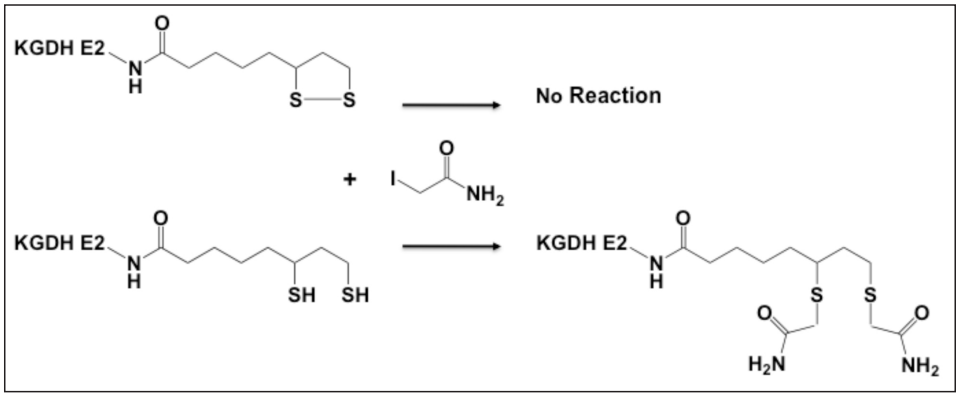
Figure 3. The Oxidation State of Lipoic Acid in the Presence of Ca^{2+} and α-Ketoglutarate. Isolated rat heart mitochondria (0.5 mg/ml) were incubated in the presence of either 0 mM or 5.0 mM α-ketoglutarate for 1 min followed by the addition of 0 μM or 20 μM Ca^{2+}. One minute after the addition of Ca^{2+}, samples were suspended in media containing iodoacetamide (100 mM) for Western blot analysis using antibody to native lipoic acid.



Scheme 1. Catalytic Mechanism of α-Ketoglutarate Dehydrogenase (KGDH).



Scheme 2. Reversible Glutathionylation of the Lipoic Acid Cofactor of KGDH.



Scheme 3. Reaction of Reduced not Oxidized Lipoic Acid with Iodoacetamide.

Ca^{2+} influx. Therefore H_2O_2-induced Ca^{2+}-stimulated inhibition of KGDH might be expected to reduce the membrane potential and thus uptake of Ca^{2+}. Future studies may determine a novel regulatory role for Ca^{2+} that relates to KGDH activity, particularly under conditions of oxidative stress. Under certain conditions, Ca^{2+}-mediated KGDH inhibition may therefore prevent excess Ca^{2+} from entering the mitochondria, thereby decreasing the likelihood of events that trigger apoptosis.

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